AMPLICON MELTING ANALYSIS WITH SATURATION DYES

FIELD OF THE INVENTION

The present invention relates to methods of performing nucleic acid analysis in the presence of a double-stranded nucleic acid binding dye.

BACKGROUND OF THE INVENTION

Methods for analyzing DNA sequence variation can be divided into two general categories: 1) genotyping for known sequence variants and 2) scanning for unknown variants. There are many methods for genotyping known sequence variants, and single step, homogeneous, closed tube methods that use fluorescent probes are available (Lay MJ, et al., Clin. Chem 1997;43:2262-7). In contrast, most scanning techniques for unknown variants require gel electrophoresis or column separation after PCR. These include single-strand conformation polymorphism (Orita O, et al., Proc Natl Acad Sci USA 1989; 86:2766-70), heteroduplex migration (Nataraj AJ, et al., Electrophoresis 1999;20:1177-85), denaturing gradient gel electrophoresis (Abrams ES, et al., Genomics 1990;7:463-75), temperature gradient gel electrophoresis (Wartell RM, et al., J Chromatogr A 1998;806:169-85), enzyme or chemical cleavage methods (Taylor GR, et al., Genet Anal 1999;14:181-6), as well as DNA sequencing. Identifying new mutations by sequencing also requires multiple steps after PCR, namely cycle sequencing and gel electrophoresis. Denaturing high-performance liquid chromatography (Xiao W, et al., Hum Mutat 2001;17:439-74) involves injecting the PCR product into a column.

Recently, homogeneous fluorescent methods have been reported for mutation scanning. SYBR® Green I (Molecular Probes, Eugene, Oregon) is a double strand-specific DNA dye often used to monitor product formation (Wittwer CT, et al., BioTechniques 1997;22:130-8) and melting temperature (Ririe KM, et al., Anal. Biochem 1997;245:154-60) in real-time PCR. The presence of heterozygous single base changes have been detected in products up to 167 bp by melting curve analysis with SYBR® Green I (Lipsky RH, et al., Clin Chem 2001;47:635-44). However, subsequent to amplification and prior to melting analysis, the PCR product was purified and high concentrations of SYBR® Green I were added. The concentration of SYBR® Green I used for detection in this method inhibits PCR (Wittwer CT, et al.,

BioTechniques 1997;22:130-1, 134-8); thus, the dye was added after amplification. A dye that could be used to detect the presence of heterozygous single base changes and could be added prior to PCR would be desirable.

Single nucleotide polymorphisms (SNPs) are by far the most common genetic variations observed in man and other species. In these polymorphisms, only a single base varies between individuals. The alteration may cause an amino acid change in a protein, alter rates of transcription, affect mRNA spicing, or have no apparent effect on cellular processes. Sometimes when the change is silent (e.g., when the amino acid it codes for does not change), SNP genotyping may still be valuable if the alteration is linked to (associated with) a unique phenotype caused by another genetic alteration.

There are many methods for genotyping SNPs. Most use PCR or other amplification techniques to amplify the template of interest. Contemporaneous or subsequent analytical techniques may be employed, including gel electrophoresis, mass spectrometry, and fluorescence. Fluorescence techniques that are homogeneous and do not require the addition of reagents after commencement of amplification or physical sampling of the reactions for analysis are attractive. Exemplary homogeneous techniques use oligonucleotide primers to locate the region of interest and fluorescent labels or dyes for signal generation. Illustrative PCR-based methods are completely closed-tubed, using a thermostable enzyme that is stable to DNA denaturation temperature, so that after heating begins, no additions are necessary.

Several closed-tube, homogeneous, fluorescent PCR methods are available to genotype SNPs. These include systems that use FRET oligonucleotide probes with two interacting chromophores (adjacent hybridization probes, TaqMan probes, Molecular Beacons, Scorpions), single oligonucleotide probes with only one fluorophore (G-quenching probes, Crockett, A. O. and C. T. Wittwer, Anal. Biochem. 2001;290:89-97 and SimpleProbes, Idaho Technology), and techniques that use a dsDNA dye instead of covalent, fluorescently-labeled oligonucleotide probes. The dye techniques are attractive because labeled oligonucleotide probes are not required, allowing for reduced design time and cost of the assays.

Two techniques for SNP typing using dsDNA dyes have been published. Allele-specific amplification in the presence of dsDNA dyes can be used to genotype with real-time PCR (Germer S, et al., Genome Research 2000;10:258-

266). In the method of the Germer reference, two allele-specific primers differ at
their 3'-base and differentially amplify one or the other allele in the presence of a
common reverse primer. While no fluorescently-labeled oligonucleotides are needed,
genotyping requires three primers and two wells for each SNP genotype. In addition,

5 a real-time PCR instrument that monitors fluorescence each cycle is necessary.

The other dye-based method does not require real-time monitoring,
needs only one well per SNP genotype, and uses melting analysis (Germer, S, et. al.,
Genome Research 1999;9:72-79). In this method, allele-specific amplification is also
used, requiring three primers, as with the previous Germer method. In addition, one

10 of the primers includes a GC-clamp tail to raise the melting temperature of one
amplicon, allowing differentiation by melting temperature in one well. Fluorescence
is monitored after PCR amplification, and real-time acquisition is not required.

SUMMARY OF THE INVENTION

15   In one aspect of the present invention, a method is provided that
requires only standard PCR reagents, primers, and the simple addition of a
"saturating" double-stranded (ds) DNA binding dye prior to PCR. For purposes of
this invention, a "saturating" dye is a dye that does not significantly inhibit PCR when
present at concentrations that provide maximum fluorescence signal for an amount of

20 dsDNA typically generated by PCR in the absence of dye, illustratively about
10ng/μL. Although the dyes are identified by their compatibility with PCR at near
saturating concentrations, it is understood that the dyes can be used at much lower
concentrations. During or subsequent to amplification, the dyes may be used to
distinguish heteroduplexes and homoduplexes by melting curve analysis in a similar

25 fashion to when labeled primers are used. The identification of heteroduplexes and
homoduplexes may be used for a variety of analyses, including mutation scanning and
SNP genotyping. The term "scanning" refers to the process in which a nucleic acid
fragment is compared to a reference nucleic acid fragment to detect the presence of
any difference in sequence. A positive answer indicating the presence of a sequence

30 difference may not necessarily reflect the exact nature of the sequence variance or its
position on the nucleic acid fragment. The term "genotyping" includes the detection
and determination of known nucleic acid sequence variances, including but not
limited to, SNPs, base deletions, base insertions, sequence duplications,

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rearrangements, inversions, base methylations, the number of short tandem repeats; and in the case of a diploid genome, whether the genome is a homozygote or a heterozygote of the sequence variance, as well as the cis/trans positional relationship of two or more sequence variances on a DNA strand (haplotyping).

5   In another aspect of this invention, various dsDNA binding dyes are identified. The dsDNA binding dyes of the present invention are capable of existing at sufficiently saturating conditions with respect to the DNA during or after amplification, while minimizing the inhibition of PCR. For example, at maximum PCR-compatible concentrations, the dsDNA binding dye has a percent saturation of at 10   least 50%. In other embodiments, the percent saturation is at least 80%, and more particularly, at least 90%. In yet other embodiments, the percent saturation is at least 99%. It is understood that the percent saturation is the percent fluorescence compared to fluorescence of the same dye at saturating concentrations, i.e., the concentration that provides the highest fluorescence intensity possible in the presence of a 15   predetermined amount of dsDNA. Illustratively, the predetermined amount of dsDNA is 100ng/10μL which is the amount of DNA produced at the end of a typical PCR at plateau. It is further understood that dye preparations may contain impurities that inhibit amplification. Such impurities should be removed prior to a determination of the percent saturation. It is also understood that the measurement of fluorescence 20   intensity for percent saturation is performed at the wavelength that is well matched for the detection of dye bound to dsDNA, and if possible, not at wavelengths that will detect high background fluorescence from free dye or secondary forms of dye binding which may occur at high dye-to-bp ratio (e.g., binding of dye to the dsDNA-dye complex or to single-stranded nucleic acids).

25   In yet another aspect of the present invention, the dsDNA binding dye has greater than 50% saturation at maximum PCR-compatible concentrations, and has excitation/emission spectra that would not suggest compatibility with standard real-time PCR instruments. "Standard" instruments for real-time PCR analysis have an excitation range of about 450-490 nm and an emission detection range of about 510-30   530 nm. It has been found that certain "blue" dyes are compatible with these systems, although their excitation/emission spectra would suggest otherwise. Thus, in this aspect of the invention a method is provided for analysis during or subsequent to PCR using a standard real-time PCR instrument and a dsDNA binding dye having an

excitation maximum in the range of 410-465 nm, more particularly in the range of

430-460 nm, and having an emission maximum in the range of 450-500 nm, more

particularly in the range of 455-485 nm, as measured in PCR buffer in the presence of

dsDNA. Suitable instrumentation may use the excitation/detection ranges above, or

5   may be modified according to the excitation/emission maxima of the dyes. Suitable

ranges for detection of the "blue" dyes of this invention as well as for detection of

traditional dyes such as fluorescein and SYBR® Green I may include 440-470 nm for

excitation and 500-560 for detection.

               In one embodiment, the dye is a dye identified as LightCycler Green

10   (or interchangeably, LC Green). Synthesis of LC Green is taught below, and the

excitation/emission spectra of LC Green are shown in Fig. 11. Additional properties

of LC Green are shown in Table 1. Similarly, other dyes identified as operative in

Table 1 may be used within the scope of this invention. While the exact structure of

some of these dyes is unknown as of yet, they are believed to be asymmetrical

15   cyanines, and various properties of these fluorescent nucleic acid dyes are shown in

Table 1.

              While the examples provided herein are directed to melting curve

analysis, it is understood that the dyes of the present invention can be used for a

variety of real-time quantitative PCR analyses, including quantification of the nucleic

20   acid, determination of initial concentration, testing for the presence of a nucleic acid,

multiplexing with labeled probes, and other PCR-based methods.

              Furthermore, while reference is made to PCR, other methods of

amplification may be compatible with the dyes of this invention. Such suitable

procedures include strand displacement amplification (SDA); nucleic acid sequence-

25   based amplification (NASBA); cascade rolling circle amplification (CRCA), Q beta

replicase mediated amplification; isothermal and chimeric primer-initiated

amplification of nucleic acids (ICAN); transcription-mediated amplification (TMA),

and the like. Therefore, when the term PCR is used, it should be understood to

include other alternative amplification methods.

30   Additionally, it is understood that the dsDNA binding dyes include

intercalators, as well as other dyes that bind to nucleic acids, as long as the dye

differentially binds to double-stranded and single-stranded nucleic acids, or otherwise

produces a differential signal based on the quantity of double-stranded nucleic acid.

Thus, the present invention includes one or more of the herein-described double-stranded binding dyes for use in quantitative or qualitative amplification analysis. In one aspect of this invention, a PCR reaction mixture is provided, comprising a target nucleic acid, PCR reagents, oligonucleotide primers

5   configured for amplifying the target nucleic acid, and a dsDNA binding dye having a percent saturation of at least 50%.

In another aspect of this invention, methods are provided for nucleic acid analysis. In one embodiment, a method of genotyping is provided comprising the steps of amplifying the target nucleic acid in the presence of a dsDNA binding dye

10   having a percent saturation of at least 50%, melting the amplified target nucleic acid to generate a melting curve, and identifying the genotype from the melting curve. In another embodiment, a method of mutation scanning is provided comprising the steps of adding a dsDNA binding dye having a percent saturation of at least 50% to a sample comprising a target nucleic acid, amplifying the target nucleic acid in the

15   presence of the dsDNA binding dye, melting the amplified target nucleic acid to generate a melting curve, repeating steps (b) and (c) on second sample to obtain a second melting curve, and comparing the melting curves. In yet another embodiment, a method of PCR analysis is provided comprising the steps of mixing a dsDNA binding dye having a percent saturation of at least 50% with a sample comprising a

20   target nucleic acid and primers configured for amplifying the target nucleic acid, amplifying the target nucleic acid in the presence of the dsDNA binding dye, and monitoring fluorescence of the dsDNA binding dye. Monitoring may occur during amplification, subsequent to amplification, or both.

In yet another aspect of this invention a method is provided comprising

25   the steps of a method of PCR analysis comprising the steps of mixing a dsDNA binding dye with a sample comprising a target nucleic acid and primers configured for amplifying the target nucleic acid, amplifying the target nucleic acid in the presence of the dsDNA binding dye, monitoring fluorescence of the dsDNA binding dye, generating a melting curve for the target nucleic acid, normalizing the melting curve,

30   repeating the mixing, amplifying, normalizing, and generating steps with at least one additional target nucleic acid, and comparing the normalized melting curves.

In an additional aspect of this invention a method is provided for nucleic acid analysis comprising the steps of mixing a target nucleic acid that is at

least partially double stranded with a dsDNA binding dye having a percent saturation
of at least 50% to form a mixture, and generating a melting curve for the target
nucleic acid by measuring fluorescence from the dsDNA binding dye as the mixture is
heated.

5   In a further aspect, kits are provided comprising amplification
reagents, oligonucleotide primers configured for amplifying the target nucleic acid,
and a dsDNA binding dye having a percent saturation of at least 50%. Any of the
dyes discussed herein may be used in the kits.

          Various dsDNA binding dyes may be used in the embodiments of this

10   invention, as described herein.

          Additional features of the present invention will become apparent to
those skilled in the art upon consideration of the following detailed description of
illustrative embodiments.

15   BRIEF DESCRIPTION OF THE DRAWINGS

          Fig. 1 shows genotyping of the Factor V Leiden using LightCycler
Green. The negative first derivative (-dF/dT) of the melting curve is shown.

          Fig. 2 shows the effect of cooling rates prior to melting analysis on the
detection of heteroduplexes.

20   Fig. 3 shows the effect of heating rates during melting analysis on the
detection of heteroduplexes.

          Fig. 4 shows a model system for detecting six combinations of
heteroduplexes.

          Figs. 5A-D show a comparison of genotyping methods; Fig. 5A shows

25   the cystic fibrosis map in which the position of an optional label on a primer is
marked (star), Fig. 5B shows genotyping using a labeled primer, Fig. 5C shows
genotyping using LightCycler Green, and Fig. 5D shows an attempt to genotype using
SYBR® Green I (Homozygotes: — - - — wt, ——— F508del,
Heterozygotes: — · — F508del, — - — I507del, - - - - F508C).

30   Fig. 6 shows genotyping using LightCycler Green on longer amplicons
(— - - — homozygote (TT), ——— homozygote (CC), — · — heterozygote (TC). The
melting curves for three individuals (not the derivatives) are shown.

Figs. 7A-B shows derivative melting curves of DNA mixtures using SYBR® Green I (Fig. 7A) and LightCycler Green (Fig. 7B).

Fig. 8 demonstrates the non-linearity of fluorescence change when multiple DNA species are present. LightCycler Green (open circles) and SYBR®

5   Green I (closed squares) are shown.

Figs. 9A-B show dye titrations to determine saturation percentages, in Fig. 9A, ◆- SYBR® Green , ■- SYBR® Gold, ▲- Pico Green, in Fig. 9B, o LightCycler Green, ■ SYTOX® Green. Illustrative PCR ranges for SYBR® Green I and LightCycler Green are indicated by the shaded box.

10   Fig. 10 illustrates the effect of dye concentrations on melting temperature.

Figs. 11A-B show the excitation and emission spectra for LightCycler Green (Fig. 11A) and SYBR® Green I (Fig. 11B).

Figs. 12A-D show high resolution melting curve analysis of

15   quadruplicate samples of six different genotypes within a 110 bp fragment of beta-globin (— - — SS, —— AA, — -- — CC, — · — SC, ·········· AC, - · - · - AS); Fig. 12A shows raw data obtained from high resolution melting of quadruplicate samples of each genotype; Fig. 12B shows normalized high resolution melting curves of the quadruplicate samples of the six genotypes; Fig. 12C shows temperature-shifted,

20   normalized, high resolution melting curves of the quadruplicate samples of the six genotypes. The samples were temperature shifted to overlay the curves between 5 and 10 % fluorescence; Fig. 12D shows fluorescence difference curves obtained from the data of Fig. 12C. Each difference curve was obtained by subtracting each sample from the normal (AA) curve to obtain the difference data. While quadruplicate samples

25   were run, due to overlap, fewer than four samples appear in some instances.

Fig. 13A shows melting curve analysis of duplicate samples of three genotypes of a 544 bp fragment of the human 5-Hydroxytryptamine receptor 2A (HTR2A) gene (— - — TC, —— CC, — -- — TT). The data have been normalized and temperature shifted using the portion between 10 and 20% fluorescence. A

30   theoretical melting map of the homoduplex is shown as Fig. 13B. The position of the single nucleotide polymorphism is marked (X).

Fig. 14 shows a difference curve of six genotypes of a 612 bp fragment of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The plots

have been normalized, temperature shifted by superimposing the portion between 30 and 40% fluorescence, and subtracted from one of the wild type plots.

Fig. 15 shows the pedigree of CEPH referenced Utah family 1331.

Genotype of HLA-A of Utah family 1331 are as follows: A:02011; B:3101; C:2402101; D:03011; E:01011. Each individual is numbered. Female (circle); male (square).

Figs. 16A and B show the melting curve of Utah family 1331 members. Six different melting curves representing six genotypes in HLA-A exon 2 exist among 17 family members. Fig. 16A shows the full melting curve and Fig. 16B shows an enlarged portion (shown in square in 16A) with the designation of genotype, and designation of individuals in parentheses.

Fig. 17 shows the determination of genotypes of two samples by mixing (——— BM15, — ·· — BM16, - - - - BM15 + BM16). Two homozygous samples BM15(0101) and BM16(0201) have a 15-bp difference on the HLA-A exon 2. The melting curve of BM15 and BM16 are similar when taken separately, but when mixed, the 15-bp mismatch shifts the melting curve.

DETAILED DESCRIPTION

SYBR® Green I is a dye extensively used for melting analysis as it shows a large change in fluorescence during PCR (Wittwer CT, et al., Biotechniques 1997;22:130-1, 134-8; Wittwer CT, et al., Real-Time PCR. In: Persing D, et al., eds. Diagnostic Molecular Microbiology: Principles and Applications. ASM Press, 2004: in press). Conceivably, such dyes could be used for both homozygous genotyping and scanning for heterozygous sequence alterations. SYBR® Green I was first used in melting analysis to distinguish different PCR products that differed in Tm by 2°C or more (Ririe KM, et al., Anal Biochem 1997;245:154-160). Subsequently, SYBR® Green I was used to identify deletions (ησhima T, et al., Clin Chem 2000;46:119-22), genotype dinucleotide repeats (Marziliano N, et al., Clin Chem 2000;46:423-5), and identify various sequence alterations (Lipsky RH, et al., Clin Chem 2001;47:635-44; Pirulli D, et al., Clin Chem 2000;46:1842-4; Tanriverdi S, et al., J Clin Microbiol. 2002;40:3237-44; Hladnik U, et al., Clin Exp Med. 2002;2:105-8). However, the Tm difference between genotypes can be small and may challenge the resolution of current instruments. Indeed, it has

been suggested that SYBR® Green I, "should not be used for routine genotyping applications" (von Ahsen N, et al., Clin Chem 2001;47:1331-1332). Melting curve genotyping with commonly used double-strand-specific DNA dyes can include an increased Tm with broadening of the melting transition (Douthart RJ, et al.,

5   Biochemistry 1973;12:214-20), and compression of the Tm difference between genotypes (Fig. 5D). These factors lower the potential of SYBR® Green I for genotype discrimination.

                Amplification of heterozygous DNA produces four different single strands that create two homoduplex and two heteroduplex products when

10   denatured and cooled. Theoretically, all four products have different Tms and the melting curve should be a composite of all four double-stranded to single-stranded transitions. However, double-strand-specific DNA dyes may redistribute during melting (Aktipis S, et al., Biochemistry 1975;14:326-31), causing release of the dye from low melting heteroduplexes and redistribution to higher melting homoduplexes.

15   Because SYBR® Green I is not saturating at concentrations compatible with PCR (Wittwer CT, et al., Biotechniques 1997;22:130-1, 134-8; Fig. 9), such redistribution is plausible and consistent with the absence of a heteroduplex transition (Fig. 5D).

                LightCycler Green and other dyes of the present invention can be used for genotyping and scanning applications. When only one PCR product is

20   amplified and the sequence is homozygous, only homoduplexes are formed. With the dyes of the present invention, Tm differences between different homoduplex genotypes are not compressed (Fig. 5C), and clear differentiation between genotypes is possible. The dyes of the present invention can also identify and distinguish multiple products present in a reaction, illustratively homoduplexes generated from

25   amplification of multiple loci or multiple targets that are homozygous. In contrast, most of the time only a few products can be observed with SYBR® Green I, presumably due to dye redistribution (see Fig. 7A).

                When one or more heterozygous targets are amplified, heteroduplex products are readily observable with the dyes of the present invention.

30   The ability to detect and identify heteroduplexes is particularly useful for detecting heterozygous genotypes as well as for scanning unknown mutations. This is not possible with conventional dsDNA dyes used in real-time PCR, such as SYBR®

Green I, SYBR® Gold, and ethidium bromide, where heteroduplex products are not observable.

Heteroduplex strands may re-associate with their perfect complement and form homoduplexes during melting. Because the concentration of products at the end of PCR is high, this re-association happens rapidly. Re-association can be minimized by limiting the time the products are near their melting temperatures, particularly between the Tms of the heteroduplex and homoduplex products. In addition to strand re-association during melting, the selective hybridization of a strand to either its perfect match, or to its mismatched complementary strand, is influenced by cooling rates. Under conditions presented herein, heteroduplex formation is most favored by rapid cooling and often disappears at rates slower than -0.1°C/s (Fig. 2). This is in contrast to denaturing HPLC techniques, where cooling rates are much slower (-0.01 to about -0.02°C/s), yet heteroduplexes are efficiently formed (Xiao W, et al., Hum Mutat 2001;17:439-74). Perhaps the relative rates of homoduplex and heteroduplex formation are strongly dependent on product size, and the results obtained using small amplicons may not be typical for the larger products more commonly used in dHPLC.

The discrimination between homozygous genotypes can be improved by melting at slower rates, at the expense of greater analysis time. One source of potential error in melting curve genotyping is the effect of DNA concentration on Tm. Using a random 100 bp amplicon of 50% GC content under PCR conditions, the difference in Tm between products at 0.05 μM and 0.5 μM is about 0.7°C (von Ahsen N, et al., Clin Chem 2001;47:1956-61; Wetmur JG, Crit Rev Biochem Mol Biol 1991;26:227-59). This change can be important when the Tms of different homozygous genotypes are very close. However, different PCR samples tend to plateau at the same product concentration, so post-amplification concentration differences are usually minimal. Also, it may be possible to estimate amplicon concentrations by real-time fluorescence and adjust the Tms for even greater genotyping precision. Alternatively, asymmetric PCR may be used to limit automatically the final concentration of PCR product.

With LightCycler Green, it is possible to distinguish all single base heterozygotes from homozygotes. In the detection of heterozygotes, the absolute melting temperature and the influence of DNA concentration are not as important as

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with methods involving differentiation between homozygous genotypes. Heteroduplexes affect the shape of the melting curve, particularly at the "early," low temperature portion of the transition. Different melting curves can be temperature matched by translating the X-axis to superimpose the "late," high temperature portion

5   of the transition. The presence or absence of heteroduplexes can then be inferred with greater accuracy.

Whatever the precision of the instrument, some genotypes will be nearly identical in Tm. One way to detect homozygous variants with the same Tm is to mix the variants together. The resulting heteroduplexes will melt at lower

10   temperatures than the homoduplexes, displayed as a drop in the normalized melting curves before the major melting transition.

Thus, using presently available PCR amplification devices, LightCycler Green can identify heteroduplexes in melting curve transitions that cannot currently be identified using SYBR® Green I. One possible reason why

15   SYBR® Green I cannot easily identify low melting transitions is shown in Fig. 7A. When several DNA fragments of increasing stability are present, the low temperature peaks are very small with SYBR® Green I compared to LightCycler Green. During melting, SYBR® Green I may be released from low temperature duplexes, only to attach to duplexes that melt at higher temperatures. This causes each successive peak

20   to be higher than the last, with the lowest temperature peaks being very small, if observable at all. As seen in Fig. 7B, Low temperature melting products are easily detected with LightCycler Green, but not by SYBR® Green I.

The advantages of using LC Green have led to identification of other dsDNA dyes that are compatible with PCR and are suited for genotyping at

25   PCR-compatible concentrations. Many of the dyes useful in the method of the present invention belong to a family of cyanines. Cyanine dyes are those dyes containing one or more divalent moieties "-C(R)=" arranged in a chain that link two nitrogen containing heterocycles. The group "R" may be hydrogen or any carbon substituent, and is illustratively hydrogen or alkyl, including C_{1-6} alkyl, which may be optionally

30   substituted. It is understood that in cyanine dyes where there is more than one divalent moiety "-C(R)=" each "R" may be selected independently. Such cyanine dyes may be monomers or dimers, as further defined by the illustrative general formulae herein described. In addition to cyanine dyes, it is contemplated herein that

other families of dsDNA binding dyes are also useful in the PCR reaction mixtures,
methods, and compositions described herein, including but not limited to
phenanthridinium intercalators and phenanthroline-based metallointercalators.

Illustrative dyes useful in the present PCR reaction mixtures, methods,
and compositions include, PO-PRO™-1, BO-PRO™-1, SYTO® 43, SYTO® 44,
SYTO® 45, SYTOX® Blue, POPO™-1, POPO™-3, BOBO™-1, BOBO™-3, LO-
PRO™-1, JO-PRO™-1, YO-PRO®-1, TO-PRO®-1, SYTO® 11, SYTO® 13, SYTO®
15, SYTO® 16, SYTO® 20, SYTO® 23, TOTO™-3, YOYO®-3 (Molecular Probes,
Inc., Eugene, OR), GelStar® (Cambrex Bio Science Rockland Inc., Rockland, ME),
thiazole orange (Aldrich Chemical Co., Milwaukee, WI) and novel dyes G5, H5, D6,
E6, P6, R6, Y6, Z6, and D8 described herein.

Illustrative cyanine dyes for use in the PCR reaction mixtures,
methods, and compositions described herein also include monomers or dimers of
unsymmetrical cyanines having pyridinium, pyrimidinium, quinolinium,
isoquinolinium, or purinium core structures, and those generally described by
Formula I:

$$Y \quad \underset{X}{\overset{(R^2)_t}{\underset{}{\bigcirc}}} \overset{R^3}{\underset{R^9}{\cdots}} \overset{R^{10}}{\underset{}{\cdots}} Q$$
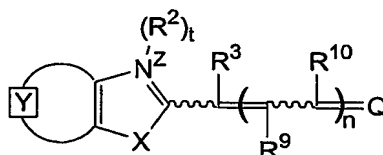
Formula I

wherein

the moiety $\boxed{Y}$ represents an optionally-substituted fused mono or
polycyclic aromatic or nitrogen-containing heteroaromatic ring;

X is oxygen, sulfur, selenium, tellurium, or a group selected from
$C(CH_3)_2$ and NR^1, where R^1 is hydrogen or alkyl, including C_{1-6} alkyl and C_{2-6} alkyl;

R^2 is alkyl, including C_{1-6} alkyl and C2-6 alkyl, cycloalkyl, including
C_{3-8} cycloalkyl, aryl, arylalkyl, including aryl(C_{1-2} alkyl), hydroxyalkyl, alkoxyalkyl,
aminoalkyl, mono and dialkylaminoalkyl, trialkylammoniumalkyl, alkyl and
arylcarbonyl, alkyl and arylcarboxamide, alkyl and arylsulfonyl, alkylenecarboxylate,
alkylenecarboxamide, alkylenesulfonate, alkylenesulfonic acid, and the like, a cyclic
heteroatom-containing moiety, or an acyclic heteroatom-containing moiety, each of
which may be optionally substituted; illustrative heteroatom-containing moieties

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include optionally substituted heteroalkyl, including methoxymethyl, ethoxyethyl, and the like, heterocyclyl, including piperidinyl, and the like, alkyl and arylsulfonates, including methylsulfonate, 4-chlorophenylsulfonate, and the like, alkoxy, including methoxy, ethoxy, and the like, amino, including methylamino, dimethylamino, and

5   the like, carbonyl derivatives, including alkyl and aryl carbonyl, alkylaminocarbonyl, alkoxycarbonyl, and the like, heteroalkenyl, including alkenylaminoalkyl, alkenyloxyalkyl, alkylaminoalkenyl, alkyloxyalkenyl, alkylideneaminoalkyl, and the like, heteroallyl, esters, amines, amides, phosphorus-oxygen, and phosphorus-sulfur bonds; and including heteroatom-containing moieties as described in U.S. Patent No.

10   5,658,751 and PCT Publication No. WO 00/66664; the disclosures of each are herein incorporated in their entirety by reference;

         t = 0 or 1;

         Z is a charge selected from 0 or 1;

         R^3, R^9, and R^{10} are each independently selected from hydrogen and

15   alkyl, including C_{1-6} alkyl and C2-6 alkyl;

         n = 0, 1, or 2; and

         Q is a heterocycle, such as a pyridinium, a pyrimidinium, a quinolinium, or a purinium, each of which may be optionally substituted.

         The term "alkyl" as used herein generally refers to a linear or

20   optionally branched hydrocarbon moiety comprising from 1 to about 12 carbon atoms, illustratively including but not limited to methyl (Me), ethyl, propyl, butyl, dodecyl, 4-ethylpentyl, and the like.

         The term "cycloalkyl" as used herein generally refers to a linear or optionally branched hydrocarbon moiety, at least a portion of which forms one or two

25   rings, comprising from 3 to about 14 carbon atoms, illustratively including but not limited to cyclopropyl, cyclopentyl, cyclohexyl, 4-methylcyclohexyl, 2,3-dimethylcyclopentyl, 3,5-dimethylcyclohexylethyl, and the like.

         The term "aryl" as used herein generally refers to a cyclic aromatic moiety, illustratively including but not limited to phenyl (Ph), naphthyl, furyl, thienyl,

30   pyrrolo, pyrazolo, isoxazolyl, isothiazolyl, oxazolyl, thiazolyl, pyridinyl, pyridazinyl, pyrimidinyl, pyrazinyl, quinolinyl, isoquinolinyl, quinoxalinyl, quinazalinyl, and the like.

The term "optionally substituted" as used herein generally refers to the optional replacement of one or more hydrogen atoms present on the parent group, including those present on carbon, nitrogen, oxygen, or sulfur atoms, with a substituent, such as halo; hydroxy; amino; thio; alkyl, cycloalkyl, haloalkyl,

5   halocycloalkyl; alkoxy, cycloalkoxy, haloalkoxy; monoalkyl and dialkylamino; aminoalkyl; monoalkyl and dialkylaminoalkyl; alkylthio; alkyl, haloalkyl, cycloalkyl, and arylcarbonyl; alkyl, haloalkyl, cycloalkyl, and arylcarbonyloxy; alkyl, haloalkyl, cycloalkyl, and arylsulfonyl; and carboxyl derivatives, such as carboxylic acids, esters, and amides. It is appreciated that the replacement of proximal hydrogen

10   atoms, including geminal and vicinal hydrogens, may be such that the substituents replacing those proximal hydrogens are taken together to form a spiro ring or a fused ring, respectively.

It is appreciated that each of the above described terms may be used in combination in chemically relevant ways to refer to other moieties, such as arylalkyl

15   referring to an aryl group as defined herein linked to an alkyl group as defined herein to form structures including, but not limited to, benzyl, phenethyl, picolinyl, 3,5-dimethoxypicolin-4-yl, and the like.

It is appreciated that the cyanine dye structures described herein may contain chiral centers. In those cases, all stereoisomers are understood to be included

20   in the description of these cyanine dye structures, unless otherwise indicated. Such stereoisomers include pure optically active isomers, racemic mixtures, and mixtures of diastereomers containing any relative amount of one or more stereoisomeric configurations.

It is also appreciated that the cyanine dye structures described herein

25   may contain geometric centers. In those cases, all geometric isomers are understood to be included in the description of the cyanine dye structures, unless otherwise indicated. Such geometric isomers include *cis*, *trans*, *E* and *Z* isomers, either in pure form or in various mixtures of geometric configurations. It is also understood that depending upon the nature of the double bond contained in the cyanine dye structures,

30   such double bond isomers may interconvert between *cis* and *trans*, or between *E* and *Z* configurations depending upon the conditions, such as solvent composition, solvent polarity, ionic strength, and the like.

It is further appreciated that when the charge Z is greater than 0, several tautomers of the compounds of Formula I may exist, including mixtures of such tautomers. Illustratively, the charge Z may be formally localized on the nitrogen atom as depicted in Formula I, or on one of the carbon atoms forming the polyene linker that connects the two heterocycles, or alternatively, the charge may be localized on the heterocycle Q. Tautomers of the charged compounds of Formula I may be depicted by rearranging the double bond-single bond configuration of compounds of Formula I, such as the illustrative structures:
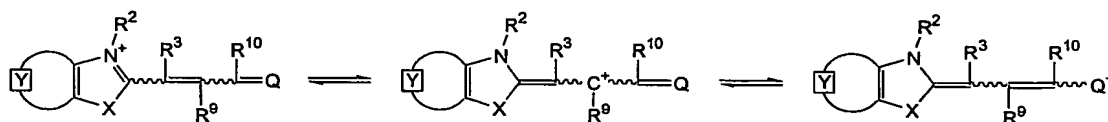
wherein $\boxed{Y}$, X, R^2, R^3, R^9, R^{10}, and Q, are as defined for Formula I, and t=1, Z=1, and n=1. The cyanine dye compounds described herein include any of the several possible tautomers, or various equilibrium mixtures of those tautomers. It is understood that the location of the formal charge is influenced by the nature of the moieties $\boxed{Y}$, X, R^2, R^3, R^9, R^{10}, and Q. It is further understood that the favored tautomer or equilibrium mixture of tautomers may depend upon conditions, such as solvent composition, solvent polarity, ionic strength, formulation, and the like. It is understood that the term "resonance structures" also refers to these various charge localizations and is equally descriptive of formulae illustrated above.

It is also understood that when compounds of Formula I carry a net charge, such as where Z is 1, or where there is present on the compounds of Formula I a charged substituent, such as an ammonium group, or a sulfonic acid group, these compounds of Formula I are accompanied by a counter ion. Any monovalent, divalent, or polyvalent counter ion is included in the description of the cyanine dye structures contained herein. Illustrative counter-ions include negatively charged counter ions such as iodide, chloride, bromide, hydroxide, oxide, acetate, trifluoroacetate, monophosphate, diphosphate, triphosphate, and the like, and positively charged counter ions such as lithium, sodium, potassium, cesium, ammonium, polyalkylammonium, and the like. Such counter ions may arise from the synthetic methods used, the purification protocol, or other ion exchange processes.

It is believed that the nature or type of counter ion does not appear to influence the functionality of the cyanine dyes described herein. It is appreciated that

when the dyes described herein are dissolved in solvents or other media used to practice the PCR reaction mixtures, methods, and compositions described herein, the accompanying counter ion may exchange with other counter ions that are present in the solvents or other media. Such additional counter ions may be solvent ions, salts,

5   buffers, and/or metals.

It is appreciated that the group R^2 may be virtually any group that arises from the nucleophilic reaction between the parent compound of Formula I, where t=Z=0:
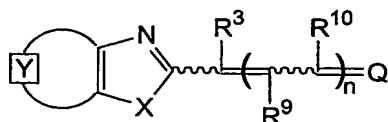
10   and a compound having the formula R^2-L, wherein L is a suitable leaving group, and R^2 is as defined above. Illustratively, R^2 is an optionally substituted alkyl, acyl, aryl, sulfonic acid, or sulfonyl group, each of which may be optionally substituted. Illustrative leaving groups L include, but are not limited to halides, such as chloride and bromide, acylates, such as acetate, formate, and trifluoroacetate, sulfonates, such

15   as methylsulfonate, trifluoromethylsulfonate, and tolylsulfonate, sulfates, such as methylsulfate, and the like.

In one illustrative embodiment, Q is an heterocycle such as, but not limited to:
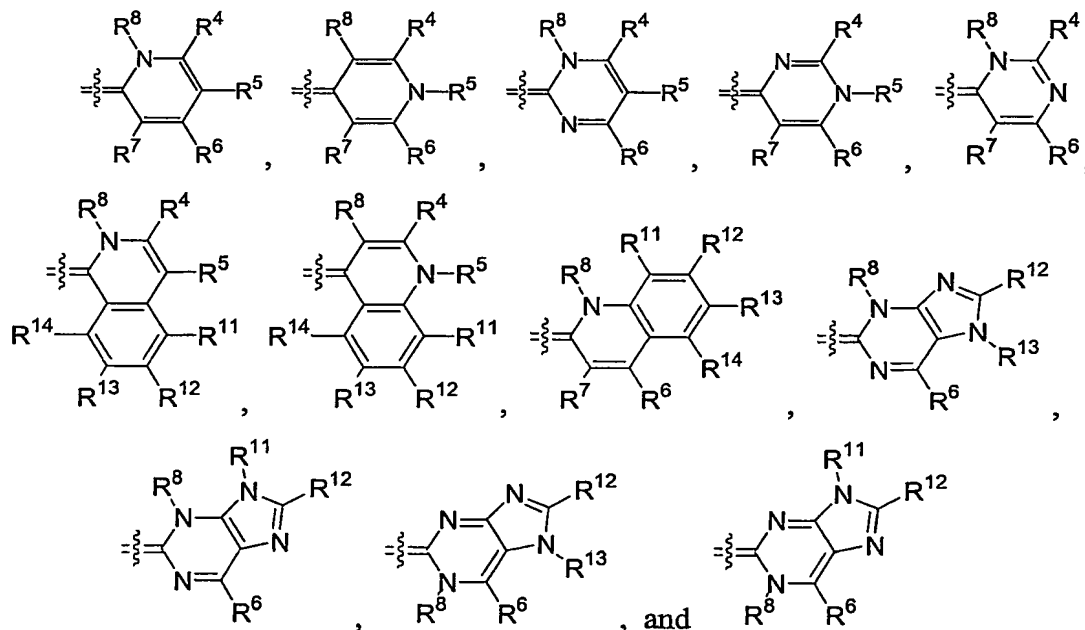
wherein R^4, R^5, R^6, R^7, R^8, R^{11}, R^{12}, R^{13}, and R^{14} are each independently selected from the group consisting of hydrogen, halogen, alkyl, cycloalkyl, heteroalkyl, heterocycloalkyl, alkenyl, polyalkenyl, alkynyl, polyalkynyl, alkenylalkynyl, aryl, heteroaryl, alkoxy, alkylthio, and dialkylamino, each of which may be optionally
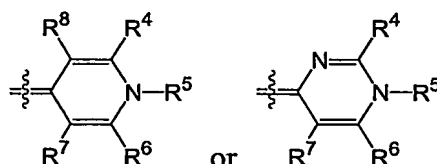
5   substituted.

In another illustrative embodiment, one of R^4, R^5, R^6, R^7, R^8, R^{11}, R^{12}, R^{13}, and R^{14} is an heteroatom-containing moiety, as described in U.S. Patent No. 5,658,751. In another illustrative embodiment, one of R^4, R^5, R^6, R^7, R^8, R^{11}, R^{12}, R^{13}, and R^{14} is a reactive group, including but not limited to halogens, hydroxy,

10   alkoxides, amines, carboxylic acids, halides, alcohols, aldehydes, thiols, alkyl, and arylthiols, alkyl and arylsulfonyls, succinimidyl esters, ketones, and isothiocyanates that may be used to attach moieties to the dye core structure, illustratively through the formation of carbon-carbon bonds, amines, amides, ethers, thioethers, disulfides, ketones, thioureas, and Schiff bases. In another illustrative embodiment, one of R^4,

15   R^5, R^6, R^7, R^8, R^{11}, R^{12}, R^{13}, and R^{14} is a BRIDGE-DYE having the formula:
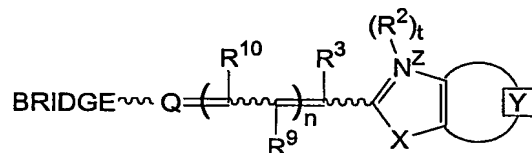
wherein Y, X, R^2, t, Z, R^3, R^9, R^{10}, Q, and n are as defined for Formula I, and BRIDGE is a single covalent bond, or a covalent linkage that is linear or branched, cyclic or heterocyclic, saturated or unsaturated, having 1-16 non-hydrogen atoms such

20   as carbon, nitrogen, phosphate, oxygen, and sulfur, such that the linkage contains any combination of akyl, ether, thioether, amine, ester, or amide bonds; single, double, triple, or aromatic carbon-carbon bonds; phosphorus-oxygen, phosphorus-sulfur, nitrogen-nitrogen, or nitrogen-oxygen bonds; or aromatic or heteroaromatic bonds. It is appreciated that in some embodiments, this dimeric structure is symmetrical about

25   BRIDGE, and in other embodiments, this dimeric structure is unsymmetrical about BRIDGE, wherein for example, any of Y, X, R^2, t, Z, R^3, R^9, R^{10}, and n are each independently selected in each occurrence on each side of BRIDGE.

Illustrative dyes for use in the present invention also include cyanine dyes of Formula I having a pyridinium or pyrimidinium core structure wherein X is

30   oxygen or sulfur; the moiety Y represents an optionally-substituted fused benzo,

optionally-substituted fused naphthaleno, optionally-substituted fused pyridino, optionally-substituted fused pyrimidino, optionally-substituted fused quinolino, and the like; n = 0 or 1; t = 0 or 1; R^2 is alkyl, such as methyl and ethyl, optionally substituted aryl, such as phenyl or tolyl, an alkylenesulfonate, such as

5   propylenesulfonic acid, or alkylsulfonyl, such as $CH_3(CH_2)_mSO_2$, where m is 0, 1, 2, or 3; and Q is an heterocycle selected from the group of structures consisting of:

wherein

      R^4 is hydrogen, alkoxy, including methoxy, ethoxy, propyloxy, and the

10   like; alkylthio, including methylthio, ethylthio, and the like; heterocyclylalkyl, including optionally substituted piperidinyl, pyrrolidinyl, piperazinyl, and the like; or heterocyclylalkyl including a charged group, including 4,4-dimethylpiperazinium-1-yl, and the like; or a reactive group, including halo, hydroxy, alkoxy, thio, alkyl and arylthio, alkyl and arylsulfonyl, amino, formyl, alkyl and arylcarbonyl, carboxyl

15   derivatives, and the like;

      R^5 is C_{1-6} alkyl, including methyl, ethyl, butyl, *sec*-butyl, isobutyl, and the like; optionally substituted phenyl; or $(CH_2)_3N^+(Me)_3$; and

      R^6, R^7, and R^8 are each independently hydrogen or methyl.

      Illustrative dyes for use herein also include cyanine dyes of Formula I

20   having a pyridinium or pyrimidinium core structure wherein X is oxygen or sulfur; the moiety $\boxed{Y}$ represents an optionally-substituted fused benzo, forming an optionally substituted benzoxazolium or benzthiazolium ring, or an optionally-substituted fused naphtho, forming an optionally substituted naphthoxazolium or naphthothiazolium ring; n = 0 or 1; t = 0 or 1; R^2 is alkyl, such as methyl, aryl, such as phenyl or tolyl, an

25   alkylenesulfonate, such as propylenesulfonic acid, or alkylsulfonyl, such as $CH_3(CH_2)_mSO_2$, where m is 0, 1, 2, or 3; and Q is a 4-pyridinium or 4-pyrimidinium heterocycle.

      Illustrative dyes for use herein also include cyanine dyes useful in the PCR reaction mixtures, methods, and compositions described herein with quinolinium

30   core structures, and generally described by Formula II:
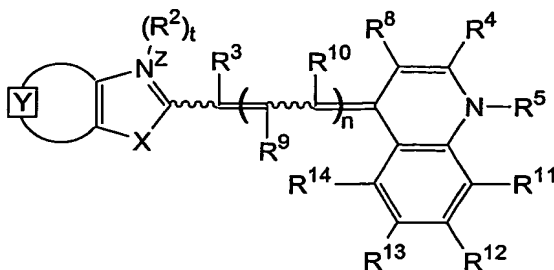
Formula II

wherein

the moiety $\boxed{Y}$ represents an optionally-substituted fused mono or polycyclic aromatic or nitrogen-containing heteroaromatic ring;

X is oxygen, sulfur, or a group selected from $C(CH_3)_2$, and NR^1, where R^1 is hydrogen or C_{1-6} alkyl;

R^2 is alkyl, including C_{1-6} alkyl and C2-6 alkyl, cycloalkyl, including C_{3-8} cycloalkyl, aryl, arylalkyl, an alkylenesulfonate, a cyclic heteroatom-containing moiety, or an acyclic heteroatom-containing moiety, each of which may be optionally substituted;

t = 0 or 1;

Z is a charge selected from 0 or 1;

R^3, R^9, and R^{10} are each independently selected from hydrogen and alkyl, including C_{1-6} alkyl;

n = 0, 1, or 2; and

R^4, R^5, R^8, R^{11}, R^{12}, R^{13}, and R^{14} are as described herein for Formula I, providing that R^4 is a moiety with a molecular weight of less than about 115, or illustratively a molecular weight of less than about 105.

Illustrative dyes for use in the present invention also include cyanine dyes of Formula II wherein the moiety $\boxed{Y}$ represents an optionally-substituted fused benzo, thereby forming a benzoxazolium or benzthiazolium ring; X is oxygen or sulfur; n = 0 or 1; t = 0 or 1; R^2 is methyl;

R^4 is hydrogen, C_{1-6} alkyl, including methyl, or optionally-substituted phenyl;

R^5 is C_{1-6} alkyl, including methyl, or optionally-substituted phenyl;

R^8 is hydrogen, and

R^{11}, R^{12}, R^{13}, and R^{14} are hydrogen or alkoxy, including methoxy.

In other embodiments, dyes for use in the present invention also illustratively include cyanine dyes of Formula II wherein the moiety $\boxed{Y}$ represents an optionally-substituted heterocycle, including 1-methylpyrido and 3-bromo-1-methylpyrido; X is oxygen or sulfur; n = 0 or 1; t = z = 0;

5 R^4 is hydrogen or C_{1-6} alkyl, including methyl;

R^5 is C_{1-6} alkyl, including methyl, optionally-substituted phenyl or heteroalkyl, including heteroalkyl having a charged group such as the group $-(CH_2)_3N(Me)_3$;

R^8 is hydrogen; and

10 R^{11}, R^{12}, R^{13}, and R^{14} are hydrogen, alkyl, including methyl, or alkoxy, including methoxy.

In another embodiment, two compounds of Formula I are taken together to form a dimer. The two compounds are linked to each other by replacing one of the substituents R^4, R^5, R^6, R^7, R^8, R^{11}, R^{12}, R^{13}, and R^{14}, as defined above,

15 present on each of the compounds of Formula I with a single divalent linker. Illustratively, two compounds of Formula I are taken together to form a dimer, where the two R^5 substituents present on the two compounds of Formula I are replaced with a single divalent linker. It is appreciated that both symmetrical and unsymmetrical dimers of Formula I compounds are contemplated herein. In the case of

20 unsymmetrical dimers of compounds of Formula I, it is understood that such asymmetry may arise by forming dimers from compounds of Formula I having different substitution patterns, or having different heterocycles Q. Further, such asymmetry may arise by forming dimers from compounds of Formula I where different substituents are replaced with the divalent linker, such as illustratively

25 replacing R^5 on a first compound of Formula I and replacing R^8 on a second compound of Formula I with the divalent linker.

In another embodiment, two compounds of Formula II are taken together to form a dimer. The two compounds are linked to each other by replacing one of the substituents R^4, R^5, R^8, R^{11}, R^{12}, R^{13}, and R^{14}, as defined above, present on

30 each of the compounds of Formula II with a single divalent linker. Illustratively, two compounds of Formula II are taken together to form a dimer, where the two R^5 substituents present on the two compounds of Formula II are replaced with a single divalent linker. It is appreciated that both symmetrical and unsymmetrical dimers of

Formula II compounds are contemplated herein. In the case of unsymmetrical dimers of compounds of Formula II, it is understood that such asymmetry may arise by forming dimers from compounds of Formula II having different substitution patterns, or having different heterocycles Q. Further, such asymmetry may arise by forming dimers from compounds of Formula II where different substituents are replaced with the divalent linker, such as illustratively replacing R^5 on a first compound of Formula II and replacing R^8 on a second compound of Formula II with the divalent linker.

The dimeric cyanine dye structures formed by compounds of Formula I may also be represented by Formula III:

$$\boxed{Y} \quad \overset{(R^2)_t}{\underset{X}{\overset{N^Z}{\diagdown}}} \quad R^3 \quad R^{10} \quad \left(\right)_n = Q\text{~~BRIDGE~~}Q' \quad R^{10'} \quad R^{3'} \quad \overset{(R^{2'})_{t'}}{\underset{X'}{\overset{N^{Z'}}{\diagup}}} \quad \boxed{Y'}$$
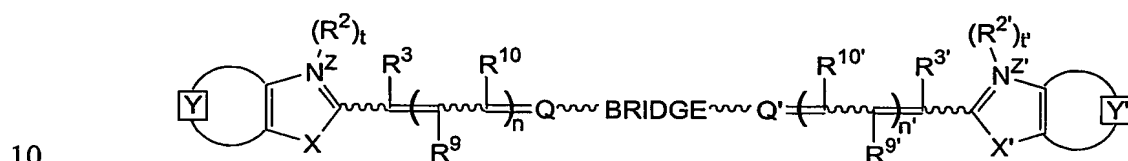
Formula III

wherein

the moieties $\boxed{Y}$ and $\boxed{Y'}$ each represent an independently selected optionally-substituted fused mono or polycyclic aromatic or nitrogen-containing heteroaromatic ring;

X and X' are each independently selected from oxygen, sulfur, selenium, tellurium, or a group selected from $C(CH_3)_2$, NR^1, or $NR^{1'}$, where R^1 and $R^{1'}$ are each independently hydrogen or C_{1-6} alkyl;

R^2 and $R^{2'}$ are each independently selected from alkyl, including C_{1-6} alkyl, cycloalkyl, including C_{3-8} cycloalkyl, aryl, arylalkyl, including aryl(C_{1-2} alkyl), a cyclic heteroatom-containing moiety, or an acyclic heteroatom-containing moiety, each of which may be optionally substituted;

t = 0 or 1;

t' = 0 or 1;

Z and Z' are each a charge independently selected from 0 or 1;

R^3, R^9, R^{10}, $R^{3'}$, $R^{9'}$, and $R^{10'}$ are each independently selected from hydrogen and alkyl, including C_{1-6} alkyl;

n = 0, 1, or 2;

n' = 0, 1, or 2;

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BRIDGE is a divalent linker comprising 2 to about 30 divalent units selected from alkylene, heteroalkylene, alkylamindiyl, alkylalkylammoniumdiyl, and the like, such as $(CH_2)_p$, $(CH_2)_pN^+Me_2(CH_2)_q$, $(CH_2)_pN^+Me_2(CH_2)_qN^+Me_2(CH_2)_r$, and the like, where p, q, and r are each independently selected from 1, 2, and 3; and

Q and Q' are heterocycles, each independently selected from the group of structures consisting of:
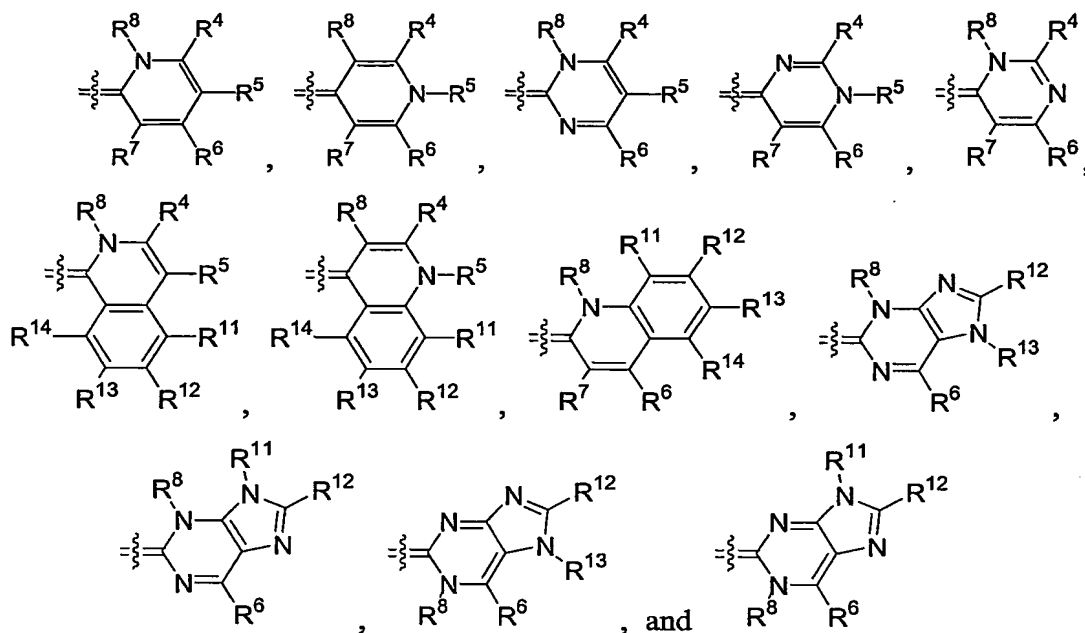
wherein R^4, R^5, R^6, R^7, R^8, R^{11}, R^{12}, R^{13}, and R^{14} are in each occurrence in compounds of Formula III independently selected from the group consisting of hydrogen, halogen, alkyl, cycloalkyl, heteroalkyl, heterocycloalkyl, alkenyl, polyalkenyl, alkynyl, polyalkynyl, alkenylalkynyl, aryl, heteroaryl, and cycloalkyl, each of which may be optionally substituted.

Illustrative cyanine dyes useful in the present PCR reaction mixtures, methods, and compositions also include, but are not limited to, LightCycler Green, PO-PRO™-1, BO-PRO™-1, SYTO® 43, SYTO® 44, SYTO® 45, SYTOX® Blue, POPO™-1, POPO™-3, BOBO™-1, BOBO™-3, and other dyes having the general Formulae IV:
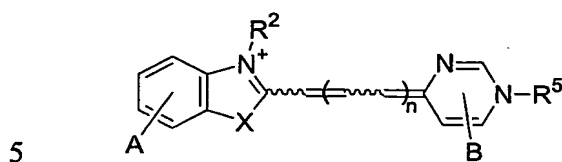
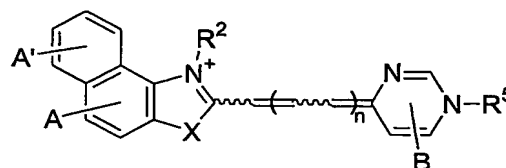-24-
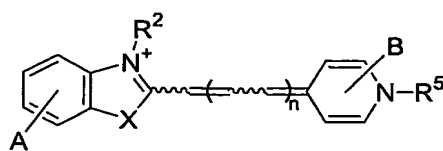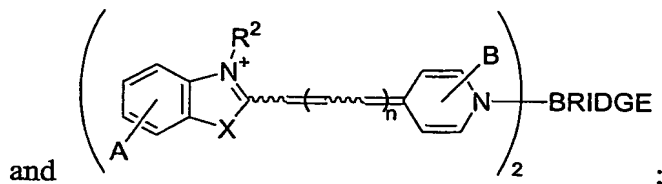
Formula IVa   Formula IVb

and the dyes G5, H5, D6, E6, P6, R6, Y6, Z6, and D8 presented in Example 14, and
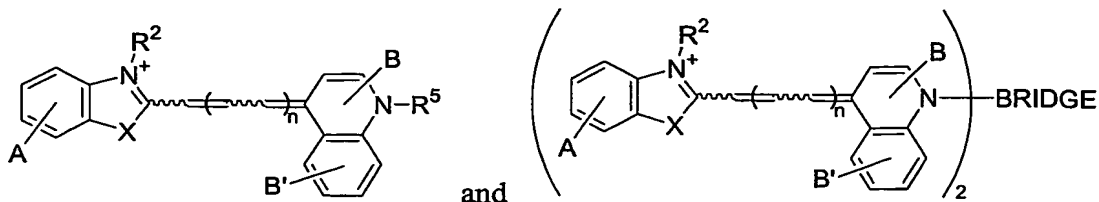other dyes having the general Formulae V:

Formula Va   and   Formula Vb

wherein n is 0, 1, or 2; R^2 is alkyl, hydroxyalkyl, alkoxyalkyl, aminoalkyl, mono and
dialkylaminoalkyl, trialkylammoniumalkyl, alkylenecarboxylate,
alkylenecarboxamide, alkylenesulfonate, and the like; R^5 is alkyl, hydroxyalkyl,
alkoxyalkyl, aminoalkyl, mono or dialkylaminoalkyl, trialkylammoniumalkyl,
alkylenecarboxylate, alkylenecarboxamide, alkylenesulfonate, optionally substituted
phenyl, and the like; X is oxygen or sulfur; A, A', and B each represent one or more
independently selected optional substituents, such as alkyl, halo, amino, haloalkyl,
alkoxy, haloalkoxy, alkyl and arylsulfonyl, haloalkylsulfonyl, alkyl and arylthio,
formyl, alkyl and arylcarbonyl, carboxyl derivatives, mono and dialkylamino,
trialkylammonium, dialkylaminoalkyl, trialkylammoniumalkyl, or a heterocycle
including pyrrolidino, piperidino, piperazino, each of which may be optionally
substituted with alkyl, amino, mono or dialkylaminoalkyl, trialkylammoniumalkyl, or
may be optionally quaternized on the nitrogen with an alkyl group, and the like; and
BRIDGE is a divalent linker having the formula $(CH_2)_pN^+Me_2(CH_2)_q$, where p and q
are independently 2 or 3, which includes the divalent linker $(CH_2)_3N^+Me_2(CH_2)_3$. It is
understood that when these dyes have a net charge, they are accompanied by one or
more counter ions, such as counter anions including halide, alkanoate, phosphate, and
the like, and counter cations including lithium, sodium, potassium, cesium,
ammonium, and the like.

Other illustrative dyes for use herein include, but are not limited to
YO-PRO®-1, TO-PRO®-1, SYTO® 11, SYTO® 13, SYTO® 15, SYTO® 16, SYTO®

20, SYTO® 23, TOTO™-3, YOYO®-3 (Molecular Probes, Inc.), GelStar® (Cambrex Bio Science Rockland Inc., Rockland, ME), thiazole orange (Aldrich), and other dyes having the general Formulae VI:

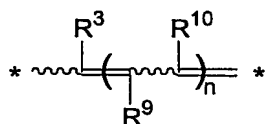5   Formula VIa   and   Formula VIb

wherein n is 0, 1, or 2; R^2 is alkyl, hydroxyalkyl, alkoxyalkyl, aminoalkyl, mono and dialkylaminoalkyl, trialkylammoniumalkyl, alkylenecarboxylate, alkylenecarboxamide, alkylenesulfonate, and the like; R^5 is alkyl, hydroxyalkyl, alkoxyalkyl, aminoalkyl, mono or dialkylaminoalkyl, trialkylammoniumalkyl,

10   alkylenecarboxylate, alkylenecarboxamide, alkylenesulfonate, optionally substituted phenyl, and the like; X is oxygen or sulfur; A, B, and B' each represent one or more independently selected optional substituents, such as alkyl, halo, amino, mono and dialkylamino, pyrrolidino, piperidino, piperazino, phenyl, hydroxy, alkoxy, thio, and alkylthio, each of which may be optionally substituted with alkyl, amino, mono or

15   dialkylaminoalkyl, trialkylammoniumalkyl, and the like; and BRIDGE is a divalent linker having the formula $(CH_2)_pN^+Me_2(CH_2)_q$, where p and q are independently 2 or 3, which includes the divalent linker $(CH_2)_3N^+Me_2(CH_2)_3$. It is understood that when these dyes have a net charge, they are accompanied by one or more counter ions, such as counter anions including halide, alkanoate, phosphate, and the like, and counter

20   cations including lithium, sodium, potassium, cesium, ammonium, and the like.

        Further, Table 1 (provided in Example 13 below) shows a comparison of several dsDNA dyes that are commonly used during or after PCR, as well as various dyes that have not previously been used for PCR analysis. Initial results have indicated that LC Green, PO-PRO™-1, JO-PRO™-1, BO-PRO™-1, G5, H5, D6, P6,

25   Y6 and D8 are quite promising dyes for heteroduplex detection. There are several surprising characteristics of these dyes. First, they do not significantly inhibit PCR at 50% saturation. In fact, saturation levels fairly close to 100% are compatible with PCR with three of these dyes. Secondly, although some of the dyes emit in the blue range, they are compatible with use in the fluorescein channel of a variety of currently

available instruments. Adjustment of the optics to better match the
excitation/emission spectra of these dyes may further improve their sensitivity for use
in quantitative or qualitative amplification analysis.

It is understood that the above cyanine dyes are illustrative, and other
cyanine dyes may be useful in the presently-described methods.

Some quinolinium-based unsymmetrical cyanines such as, but not
limited to, SYBR® Green I, SYTOX® Green, SYTO® 14, SYTO® 21, SYTO® 24,
SYTO® 25, TOTO™-1 and YOYO®-1 have not proven useful for heteroduplex
detection or for the detection of multiple products in a closed-tube system. When the
dye is a monomer of a quinolinium-based cyanine, it is possible that bulky
substitutions on the carbon next to the nitrogen of the quinolonium ring (position
equivalent to R^4) interfere with the dye's ability to function in the methods of the
present invention. Bulky substitutions are, for example, long-chain branched hetero-
atom-containing aliphatic or aromatic moieties substituted with branched-chain
aliphatic moieties that are larger than MW of about 105. This restriction, however,
does not apply to any of the pyridinium or pyrimidinium cyanines mentioned earlier.
In the case of quinolinium-based cyanine dimers, the distance between the left and
right ring systems, as defined by the divalent fragment:

$$* \sim\!\!\sim\!\!\sim\overset{\displaystyle R^3}{\underset{\displaystyle \underset{R^9}{|}}{|}}\!\!\left(\!\!\sim\!\!\sim\!\!\right)_{\!n}\!\!\overset{\displaystyle R^{10}}{|} \; *$$

also appears to determine functionality. Functionality may be determined by
heteroduplex detection, as taught herein in Examples 13-14. Other dyes previously
described as useful in real-time monitoring of PCR, such as SYBR® Gold, Pico
Green, and ethidium bromide have also been shown to be ineffective in heteroduplex
detection in a closed-tube PCR system.

The dyes for use in the present invention may be used in a dye-based
method for SNP genotyping, requiring only two unlabeled oligonucleotide primers
and one well for each SNP genotype, and not requiring real-time PCR. A dsDNA dye
is used such that heterozygotes are identified by the presence of heteroduplexes that
alter the shape of the post-amplification melting curve. Different homozygous
genotypes are differentiated by their Tm difference, or alternately by mixing a known
homozygous DNA sample with the unknown and looking for heteroduplexes.

Illustratively, PCR primer design is greatly simplified because very short amplicons can be used, preferably immediately flanking the SNP. Such short amplicons also amplify very efficiently, reduce the risk of amplifying alternate targets, and allow very rapid thermal cycling.

5   The design of PCR primers is not an exact science, and often trial and error is necessary. Although some rules for PCR primer design are generally accepted, the validity of these rules has not been tested. Because the effect of different genotypes on melting curves is greater with short amplicons, short amplicons are preferred (≤ 100 bp), and the shortest possible amplicons are often best (≤ 50 bp).

10   Therefore, to design primers for genotyping with dsDNA dyes, one illustratively starts with each flanking primer right next to the SNP position. That is, the amplicon length will be the length of primer 1, plus the length of primer 2, plus the length of the region that needs to be tested (the length of an SNP is 1). For efficient amplification, the melting temperature (Tm) of the two primers should be nearly the same. Convenient

15   Tms for primers may be 50 to 70 degrees C. Primers with the highest Tm illustratively will allow the fastest thermal cycling, while primers with lower Tm are generally less expensive and produce the shortest amplicons, resulting in greater genotyping differences. Primer lengths between 12 and 30 bases are usually used. Illustratively, each primer is built away from the SNP until the calculated Tm is

20   closest to the desired Tm. Methods for Tm calculation are well known in the art (e.g., Clin. Chem. 2001;47:1956-61). In general, the primer lengths will not be the same when the Tms are matched as closely as possible. For example, the primer lengths used in the Factor V SNP assay (Fig. 1) are 17 and 24 bases long both with a calculated matched Tm near 62°C.

25   Thermal cycling parameters for amplification can be very short because little primer extension is required for such short amplicons. After an initial denaturation of genomic DNA before thermal cycling, denaturation and annealing temperatures do not need to be held, and the extension time can be 10 s or less. It is even possible to reduce the programmed extension time to zero, allowing each cycle

30   to be performed in less than 20 s. Alternately, an extension time of 1 s can be used. Because the amplicon is so short, large amounts of polymerase are not required (<0.6 units per 10 μl may be used).

Thus, the following illustrative steps may be followed for SNP genotyping according to the present invention:

1.   Choose a target Tm and start with the 3'-end of each primer right next to the SNP position. Optionally, one primer may be shifted slightly away from the SNP position to avoid 3' complementarity between primers to decrease the risk of primer dimer formation.

2.   Design each primer outward until the calculated Tm is as close as possible to the target Tm.

3.   Rapidly thermal cycle the sample in the presence of PCR reagents and a dsDNA dye that allows heteroduplex detection.

4.   Form heteroduplexes by rapid cooling at a rate of at least -0.1°C/s, preferably at least -2°C/s, and most preferably at least -5°C/s after denaturation.

5.   Heat at 0.1 to 0.5°C/s and acquire a melting curve.

6.   If the amplification fails, move the 3'-end of one of the primers out 1 base and repeat all steps until successful.

In an illustrated example, all heterozygotes can be detected by the effect of the heteroduplexes on the melting curve (Fig. 4). In addition, 4 out of 6 homozygous differences (A vs C, A vs G, C vs T, and G vs T) are very easily distinguished by Tm shifts (Fig. 4, arrows). However, to distinguish A vs T homozygotes, high resolution melting may be necessary, and in some cases, C vs G homozygotes cannot be differentiated even with high resolution melting. In the cases where differentiation of homozygotes is difficult, a sample of a known homozygous genotype may be mixed in roughly equal amounts with the unknown genotype either before or after amplification. The mixture is amplified (if not previously amplified), denatured, and melted. If the genotypes are the same, the melting curve of the mixture will be the same as the melting curve of the known homozygous genotype. If the genotypes are different, heteroduplexes will be produced and identified by an altered shape of the melting curve. Illustratively, small amplicons may be used when genotyping for known sequence variants. Large amplicons may be preferred when scanning for unknown variants.

Unsymmetrical cyanine dyes can be prepared by a general method that attaches the benzazolium portion of the molecule to the pyridinium (or quinolinium,

pyrimidinium, purinium) portion through one or more "-C(R)=" groups. As described
in U.S. Patent No. 5,436,134 and references cited therein, the number of "-C(R)="
groups is determined by the specific synthetic reagents used in the synthesis. In the
synthesis of monomethine dyes (R=H, n=0) such as LC Green, a combination of
5   reagents is used in which the methine carbon atom results from either A on the
benzazolium salt or B on the pyridinium salt being methyl and the other of A or B
being a reactive leaving group that is typically methylthio, methylsulfonyl, or chloro,
but which can be any leaving group that provides sufficient reactivity to complete the
reaction. One possible way to prepare LC Green and other similar dyes is as follows:
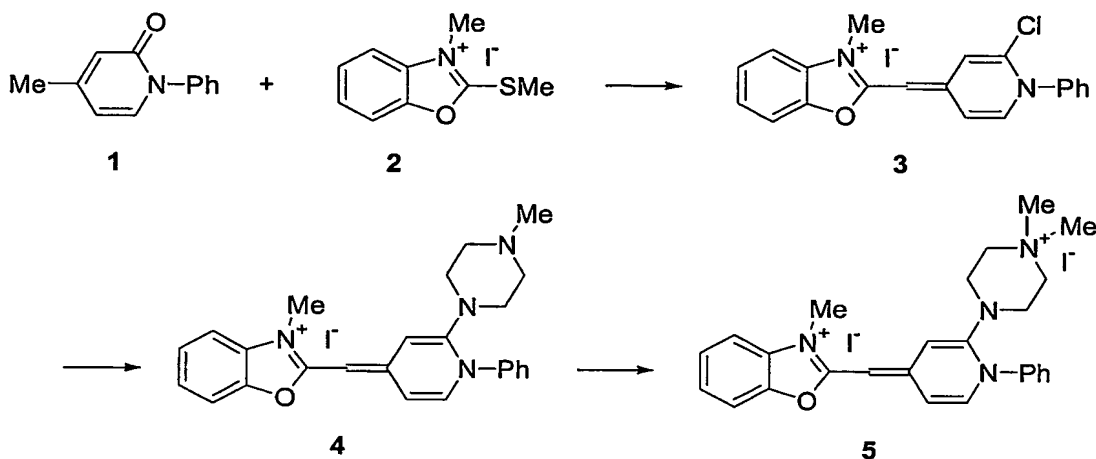
10

The starting material, Compound 1 is prepared by heating 4-methyl-2-
pyridinone (Aldrich) to reflux with copper powder, potassium carbonate and
iodobenzene for 48 hours. The reaction is cooled to room temperature, partitioned
between water and ethyl acetate, filtered, and the organic layer is dried over
15   magnesium sulfate. The crude product is purified on a silica gel column, eluting with
1:1 ethyl acetate/hexanes to yield Compound 1.

Another starting material, Compound 2, is prepared by adding
2-(methylthio)benzoxazole to methyl iodide in DMF and heating in a sealed tube at
150°C for one hour to obtain Compound 2, as the iodide salt.

A mixture of Compound 1, phosphorous oxychloride, and a catalytic
amount of DMF in methylene chloride is heated to reflux for 24 hours. The mixture
is cooled to room temperature and another volume of methylene chloride is added,
followed by Compound 2 and one equivalent of triethylamine. The mixture is stirred
at room temperature for 6 hours. A solid is separated by filtration and purified using a
25   silica gel column eluting with a mixture of ethyl acetate/chloroform/methanol. The

purified compound is then redissolved in methanol and added to an excess of sodium

iodide in water. Compound 3 is isolated by filtration as the iodide salt and dried in

vacuo.

Compound 3 is then mixed with 1-methylpiperazine in 1,2-

5   dichloroethane and heated at 55°C for 2 hours. The resulting product (Compound 4)

is then quaternized by adding an excess of methyl iodide and Proton Sponge

(Aldrich), and is expected to yield LightCycler Green (Compound 5) as the diiodide

salt.

EXAMPLE 1

10   PCR Protocol

Labeled and unlabeled oligonucleotides were obtained from IT

Biochem (Salt Lake City, UT), Qiagen Operon (Alameda, CA), or Synthegen

(Houston, TX). PCR was performed in 10 μl volumes in a LightCycler® (Roche

Applied Systems, Indianapolis, IN) with programmed transitions of 20°C/s unless

15   otherwise indicated. The amplification mixture included 50 ng of genomic DNA as

template, 200 μM of each dNTP, 3 mM MgCl$_2$, 100 mM 2-amino-2-methyl-1, 3-

propanediol, pH 8.8, 0.04 U/μl Taq polymerase (Roche), 500 μg/ml bovine serum

albumin, and 0.5 μM of each primer unless indicated otherwise. Genotyped human

genomic DNA was obtained from prior studies (Gundry CN, et al., Genetic Testing,

20   1999;3:365-70; Herrmann M, et al., Clin Chem 2000;46:425-8) or from Coriell Cell

Repositories (Camden, NJ). LightCycler Green was included in the PCR reaction at

10 μM unless otherwise indicated. When SYBR® Green I was used as the indicator, a

1:10,000 final dilution from the Molecular Probes stock was used. The dye is added

before PCR, amplification performed, and the melting transition of the amplicon is

25   monitored on the LightCycler® or by high resolution melting analysis. Different

homozygotes are distinguished by amplicon melting temperature (Tm).

Heterozygotes are identified by low temperature melting of heteroduplexes that

broaden the overall melting transition. Melting analysis requires about 1 min and no

sample processing is needed after PCR.

30   To study the sensitivity of LC Green, SYBR® Green I, and other

dsDNA binding dyes, polymorphisms in Factor V Leiden, cystic fibrosis (F508del,

F508C, I507del, I506V), and HTR2A (T102C) genes were analyzed. In addition,

engineered plasmids were used to systematically study all possible single base

changes. Heteroduplexes produced by amplification of heterozygous DNA were best detected by rapid cooling (at least - 2°C/s) of denatured products, followed by rapid heating during melting analysis (0.2 to 0.4 °C/s). All heterozygotes were distinguished from homozygotes by a broader melting transition. Different

5   homozygotes could often be distinguished by their Tm. Homozygotes with an A to T base change could only be distinguished by high resolution melting analysis or by mixing homozygotes. Homozygous G to C base changes could not reproducibly be distinguished, even with high resolution analysis, without mixing homozygotes. The amplicons varied in length from 44 to 331 bp.

10   While LC Green is used in the Examples provided herein, it is understood that other dyes according to this invention may be used.

EXAMPLE 2

Melting Curve Analysis

Melting analysis was performed either on the LightCycler®

15   immediately after cycling, or subsequently on a high-resolution melting instrument (HR-1, Idaho Technology, Salt Lake City, UT). However, it is understood that melting curve analysis may be performed in the absence of amplification. When the LightCycler® was used, the samples were first heated to 94°C, cooled to 60°C at a program setting of -20°C/s, then melted at 0.2°C/s with continuous acquisition of

20   fluorescence. For high-resolution melting, the samples were first amplified in the LightCycler®, then heated momentarily in the LightCycler® to 94°C and rapidly cooled (program setting of -20°C/s) to 40°C unless stated otherwise. The LightCycler® capillaries were then transferred one at a time to the high-resolution instrument and heated at 0.3°C/s unless otherwise stated. The HR-1 is a single sample

25   instrument that surrounds one LightCycler® capillary with an aluminum cylinder. The system is heated by Joule heating through a coil wound around the outside of the cylinder. Sample temperature is monitored with a thermocouple also placed within the cylinder and converted to a 16-bit digital signal. Fluorescence is monitored by epi-illumination of the capillary tip (Wittwer CT, et al., BioTechniques 1997;22:176-

30   81) that is positioned at the bottom of the cylinder and also converted to a 16-bit signal. Approximately 50 data points are acquired for every °C.

In some cases it is advantageous not to denature the product after PCR before melting curve acquisition. For example, when the goal is to type the number

of repeat sequences (e.g., STRs, VNTRs), amplification may be stopped at the
extension step during the exponential phase of the reaction before plateau, and then
melting analysis is performed. This way, homoduplex extension products can be
analyzed. In repeat typing, homoduplex products can be more informative than

5   heteroduplex products, especially since many different heteroduplex products may
form from different alignment of the repeats. In some cases, it may be helpful to
obtain both a homoduplex melting curve (without prior denaturation) and a
heteroduplex melting curve (with denaturation and the formation of all possible
duplex combinations). The difference between these two melting curves gives a

10   measure of the extent of heteroduplexes that can be formed, using the same sample as
the "homoduplex control".

            LightCycler® and high-resolution melting data were analyzed with
custom software written in LabView. Fluorescence vs temperature plots were
normalized between 0 and 100 percent by first defining linear baselines before and

15   after the melting transition of each sample. Within each sample, the fluorescence of
each acquisition was calculated as the percent fluorescence between the top and
bottom baselines at the acquisition temperature. In some cases, derivative melting
curve plots were calculated from the Savitsky-Golay polynomials at each point (Press
WH, et al., eds. Numerical recipes in C, 2nd ed. New York: Cambridge University

20   Press, 1992:650-5). Savitsky-Golay analysis used a second-degree polynomial and a
data window including all points within a 1°C interval. Peak areas and melting
temperatures were obtained by using non-linear least squares regression to fit multiple
Gaussians. In some cases, the X-axis for each normalized melting curve was
translated so that the tracings overlapped within a certain fluorescence range. This

25   "temperature shifting" corrects for any minor inter-run temperature variation and
increases the ability to distinguish heterozygotes from homozygotes. The difference
between genotypes can also be magnified by plotting the fluorescence difference
between genotypes at each temperature.

                                    EXAMPLE 3

30   Single Nucleotide Polymorphism Genotyping with LightCycler Green:
                    Genotyping the Factor V Leiden Mutation
                A 43 bp amplicon was formed from primers 18 and 24 bases in length,
immediately flanking the location of the factor V Leiden mutation. Both primers had

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an estimated Tm of 62°C. The samples were cycled 35 times with the following
protocol: 94°C with no hold, 60°C with no hold, and 72°C with a 10 s hold. After
amplification, the samples were heated momentarily in the LightCycler® to 94°C,
cooled rapidly (program setting of –20°C/s) to 60°C, and PCR products melted at
5   0.2°C/s with continuous fluorescence acquisition.

        Derivative melting curves of PCR products amplified from different
genotypes at the Leiden locus of the factor V gene are shown in Fig. 1. LightCycler
Green was used for fluorescent monitoring of the melting transition between double-
and single-stranded products. The Leiden mutation is located 19 bases from one end
10   of the amplicon. Results from ten homozygous wild type, two heterozygous, and one
homozygous Leiden genotypes are shown. The amplicon melting temperature of the
homozygous mutant is about 1°C less than the homozygous wild type melting
temperature. Heterozygous samples show a secondary, low temperature melting
transition attributable to heteroduplex formation. A similar experiment using SYBR®
15   Green I failed to detect this secondary melting transition in heterozygotes (data not
shown).

        The effects of cooling rate and heating rate were studied using
heterozygous factor V Leiden DNA on the LightCycler®. To study the effect of
cooling rate, the samples were amplified as above, heated to 85°C, and then cooled
20   from 85°C to 60°C at rate of -20, -2, -1, -0.5, or -0.1°C/s, followed by a constant
heating rate of 0.2 °C/s for melting curve acquisition. Rapid cooling was necessary
for significant heteroduplex formation (Fig. 2). Heteroduplexes were not observed
when the cooling rate was -0.1°C/s or slower. The greatest heteroduplex formation
occurred when capillary samples were rapidly transferred from boiling water to ice
25   water (data not shown). With cooling on the LightCycler®, heteroduplex formation
appeared to plateau at programmed rates faster than -5°C/s (Fig. 2). However,
measurement of actual sample temperatures showed that the cooling rate increased
only, slightly with programmed rates faster than -5°C/s: when the instrument was
programmed to cool at -20°C/s, the actual rate was about - 6°C/s.

30   The effect of heating rate was studied by cooling at a programmed rate
of -20°C/s, followed by melting at 0.05, 0.1, 0.3, or 0.5°C/s. The relative percentage
of observed heteroduplexes was greater with higher heating rates (Fig. 3). The
apparent Tm also shifts to higher temperatures as the rate increases and the melting

process deviates more from equilibrium (Gundry CN, et al., Genetic Testing, 1999;3:365-70).

EXAMPLE 4

Systematic Study of SNP Genotyping with Plasmids

5   Engineered plasmids were used for systematic study of melting curve genotyping of all possible single base changes. The plasmids (DNA Toolbox, Cambrex Bio Science Rockland Inc.) contained either A, C, G, or T at a defined position amid 40% GC content (Highsmith WE, et al., Electrophoresis 1999;20:1186-94). Primers with a Tm of 62 +/- 1°C were immediately adjacent to the polymorphic

10   position. The DNA templates were used at 10^5 copies and PCR was performed with 35 cycles of 94°C with no hold, 60°C with no hold, and 75°C for 5 s. The LightCycler® was used for melting analysis.

Fig. 4 demonstrates that all possible heteroduplexes formed from single base polymorphisms can be distinguished from the homoduplex samples. In

15   each case, the presence of heteroduplexes results in a low temperature shoulder on derivative melting curve plots. When the sample includes only homoduplexes formed from the amplification of homozygotes, no low temperature shoulders are present. Furthermore, AA or TT homozygotes are clearly distinguished from CC or GG homozygotes by their melting temperature. It is not clear from these "low-resolution"

20   plots (obtained on the LightCycler®) whether all heterozygotes can be distinguished from each other, or whether AA can be differentiated from TT, and CC can be differentiated from GG. However, high-resolution data (not shown) demonstrates that AA can be distinguished from TT, and most (if not all) heterozygotes can be distinguished. The stability of CC and GG homozygotes appears to be very similar

25   and any difference is difficult to resolve on current instrumentation, without mixing homozygotes.

EXAMPLE 5

Genotyping of the cystic fibrosis gene with labeled primers:
LightCycler Green or SYBR® Green I

30   KlenTaq1 polymerase (0.04 U/ μl, AB Peptides, St. Louis, MO), 88 ng of TaqStart antibody (ClonTech, Palo Alto, CA), and 50 mM Tris, pH 8.3 were used in PCR instead of Taq polymerase and 2-amino-2-methyl-1, 3-propanediol. A 44 bp fragment was amplified with the primers GGCACCATTAAAGAAAATAT (SEQ ID

NO 1) and TCATCATAGGAAACACCA (SEQ ID NO 2). The first primer was either 5'-labeled with Oregon Green, or the reaction was performed in the presence of SYBR® Green I or LightCycler Green. The primers flank the mutational hot spot containing the F508del, I507del, and F508C variants. PCR was performed through 40 cycles of 85°C and 58°C (0 s holds). Six samples were monitored during melting curve acquisition on the LightCycler®.

Derivative melting curves of PCR products amplified from different genotypes at the I507/F508 region of the cystic fibrosis gene are shown in Figs. 5B-D. The PCR products were 41 or 44 bases long (Fig. 5A). Either a 5'-labeled primer (Fig. 5B), LightCycler Green (Fig. 5C), or SYBR® Green I (Fig. 5D) was used for fluorescent monitoring of the melting transition between double and single stranded products. Results from two homozygous and three heterozygous genotypes are shown.

The duplex stability of the different genotypes follows theoretical calculations (von Ahsen N, et al., Clin Chem 2001;47:1956-61), with F508del ~ I507del < Wild type < F508C. Except for F508del and I507del, the genotypes are distinguishable by the Tms of their major transitions. The standard deviation of the Tm of 10 replicate wild type samples was 0.12 °C when melted on the LightCycler®. When melted on the high-resolution instrument, the standard deviation of the Tm of the same 10 samples was 0.04 °C.

When a heterozygous sample is amplified by PCR, two homoduplex and two heteroduplex products are expected (Nataraj AJ, et al., Electrophoresis 1999;20:1177-85). However, when SYBR® Green I was used as the fluorescent indicator, only a single melting peak was apparent for each genotype (Fig. 5D). In contrast, when labeled primers or LightCycler Green are used under the same conditions, two clearly defined peaks appeared (Figs. 5B and 5C). The lower temperature peak is always smaller than the higher temperature peak, and presumably indicates the melting transition of one or both heteroduplex products. As might be expected, the heterozygotes with 3 bp deleted (F508del and I507del) resulted in heteroduplex peaks that were more destabilized than heteroduplex peaks from a single base change (F508C). The primary peak from the F508C heterozygote was at a higher temperature than wild type, reflecting the greater stability of the T to G transversion (Gundry CN, et al., Genetic Testing, 1999;3:365-70).

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EXAMPLE 6

Mutation scanning with LC Green

The HTR2A single nucleotide polymorphism was studied. The PCR was performed with KlenTaq, TaqStart, and Tris as described for the cystic fibrosis locus. A 331 bp fragment of the hydroxytryptamine receptor 2A (HTR2A) gene included the common polymorphism (T102C) within exon 1 (Lipsky RH, et al., Clin Chem 2001;47:635-44). The reaction was cycled 40 times between 95°C with no hold, 62°C with a 2 s hold, and 74°C with a 20 s hold. A high-resolution melting curve was obtained.

Fig. 6 demonstrates that LightCycler Green can be used to scan for sequence variants. That is, the location of the sequence variant need not be known. The presence of any variant can be detected within a large amplicon. As seen in Fig. 6, all three genotypes of the single nucleotide polymorphism in the HTR2A gene (homozygous T, homozygous C and heterozygous T/C) can be differentiated within a 331 bp amplicon. Melting curve precision and the ability to distinguish different genotypes depends on the temperature and fluorescence resolution of the instrument.

EXAMPLE 7

Melting curve analysis of a DNA size ladder:

Comparison of SYBR® Green I to LightCycler Green

One hundred ng of a DNA size ladder (Low Mass DNA Ladder, Gibco BRL) having six distinct dsDNA species was mixed with either SYBR® Green I (1:10,000) or LightCycler Green (10 μM) in 3 mM $MgCl_2$, 100 mM 2-amino-2-methyl-1, 3-propanediol, pH 8.7 buffer. A melting curve was obtained on the high-resolution instrument at 0.1°C/s.

As discussed above, LightCycler Green, unlike SYBR® Green I, can identify heteroduplexes in melting curve transitions at concentrations compatible with PCR. One reason why SYBR® Green I cannot easily identify low melting transitions is illustrated in Fig. 7. When several DNA fragments of increasing stability are present, the low temperature peaks are very small with SYBR® Green I as compared to LightCycler Green. One explanation is that during melting, SYBR® Green I may be released from low temperature duplexes, only to attach to duplexes that melt at higher temperatures. This causes each successive peak to be higher than the last, with the lowest temperature peaks being very small, if observable at all. LightCycler

Green, which is present at a much higher saturation level, has visible peaks for even low temperature duplexes. While LC Green was present at near saturation levels in this example, surprisingly, LC Green can detect the low temperature peaks when diluted to saturation levels of 5-20%. For example, the data illustrated in Fig. 13 were obtained using an LC Green concentration of 1 μM. Thus, while the mechanism is not understood, LC Green and various other saturating dyes of this invention do not appear to redistribute during melting.

If the areas of each peak in Fig. 7 are determined and divided by the known amount of each of the DNA species, the relative sensitivity for each DNA species can be assessed (Fig. 8). As shown in Fig. 8, with LightCycler Green, low temperature melting peaks are favored, whereas with SYBR® Green I, a large enhancement of signal is observed at high temperature.

EXAMPLE 8

Titration curves of common dsDNA dyes

And determination of useful concentration range of LightCycler Green in PCR

One hundred ng of the low mass DNA ladder was mixed with different concentrations of common dsDNA dyes in the presence of 3 mM $MgCl_2$, 50 mM Tris, pH 8.3, 250 μg/ml BSA and 200 μM each dNTP in a final volume of 10 μl. The samples were transferred to LightCycler® tubes and the fluorescence measured at 40°C on the real-time fluorimeter. The fluorescence was normalized to the maximum fluorescence obtained with each particular dye.

Dilution studies were done using a 152 bp HTR2A amplicon in 10 μl volumes with 3 mM Mg^{2+}, 50 mM Tris-HCl, pH = 8.3, 500 μg/ml BSA, 200 μM each dNTP, 0.5 μM of each primer, 50 ng genomic DNA, 0.4 U of Taq Polymerase, and 88 ng of TaqStart antibody, with LC Green dilutions ranging from 2 μM to 100 μM. After an initial denaturation for 10 s at 95°C, 40 cycles of 95 °C for 0 sec, 62 °C for 2 sec, and 72 °C for 20 sec were performed. After additional temperature conditioning on the LightCycler® (95 °C for 0 s, 55 °C for 0 s) the samples were melted on the high-resolution instrument with a slope of 0.3 °C/sec.

Figs. 9A-B show the concentrations of SYBR® Green I and LC Green that are compatible with PCR. At concentrations compatible with PCR, SYBR® Green I is far from saturating the amount of DNA typically present at the end of PCR. LightCycler Green, in contrast, can be used over a wide range of concentrations,

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including those that are saturating. Typical melting curves over a 50-fold range of
LightCycler Green concentration are shown in Fig. 10.

EXAMPLE 9

Fluorescence spectra of SYBR® Green I and LightCycler Green

5   The excitation and emission spectra of SYBR® Green I and
LightCycler Green bound to DNA were measured on a Photon Technology
fluorimeter (FL-1). LightCycler Green (10 μM) or SYBR® Green I (1:10,000) was
added to 100 ng DNA (Low Mass DNA Ladder) in the presence of 3 mM MgCl$_2$, 50
mM Tris, pH 8.3, 250 μg/ml BSA and 200 μM each dNTP in a final volume of 60 μl.

10   LightCycler® optics are well matched to SYBR® Green I excitation and
emission (Fig. 11). Even though LightCycler Green is poorly matched to
LightCycler® optics, the fluorescence signal observed on the LightCycler® with
LightCycler Green at some PCR-compatible concentrations is greater than that
usually observed from SYBR® Green I (data not shown).

15   Example 10

Genotyping of beta-globin gene using X-axis adjustment

and fluorescence difference analysis

           A 110 bp fragment was amplified from the human beta globin region
on chromosome 11 (accession# NG_000007). The 110 bp product included the sites

20   of the common beta-globin mutations HbS and HbC. DNA was extracted from dried
blood spots of 4 different individuals of each common genotype. The genotypes
included 3 homozygous (AA, SS, and CC) and 3 heterozygous (AS, AC, and SC)
types. The forward and reverse primers were ACACAACTGTGTTCACTAGC (SEQ
ID NO 3) and CAACTTCATCCACGTTCACC (SEQ ID NO 4), respectively. Each

25   10 μl reaction contained 50 μg of genomic DNA, 0.50 μM each primer, 10 μM LC
Green, 3 mM MgCl$_2$, 50 mM Tris, pH 8.3, 500 μg/ml bovine serum albumin, 0.2 mM
each dNTPs, 0.04U/μl Klentaq™ (AB Peptides, St. Louis, MO), 88 ng TaqStart™
antibody (CloneTech, Palo Alto, CA). PCR reaction conditions were as follows: one
pre-cycling denaturation at 95° C for 5 sec; 35 cycles of 94° C for 0 sec, 50° C for 2

30   sec, 72° C for 2 sec with a slope of 2° C per second. Single fluorescence acquisitions
were taken for each sample after the 2 sec extension. After PCR amplification, the
samples were cooled at a programmed rate of -20° C/sec. Immediately following the
rapid cooling, melting was performed on a custom 16-bit high resolution melting

instrument from 70° C to 93° C at a rate of 0.30° C/sec while continuously acquiring
fluorescence.

High resolution melting curve data are obtained by measuring
fluorescence as the temperature of the sample is increased. The original data from
quadruplicate samples of 6 genotypes of beta-globin are shown in Fig. 12A. Note that
the magnitude of the fluorescence is variable between different samples because of
sample volume differences and variable capillary optics.

Magnitude differences between samples can be normalized by using
linear baselines of each curve before and after the major transition. Specifically, two
linear regions are selected, one before and one after the major transition. These
regions define two lines for each curve, an upper 100% fluorescence line and a lower,
0% fluorescence line. The percent fluorescence within the transition (between the
two regions) is calculated at each temperature as the percent distance between the
extrapolated upper and lower lines. The normalized result for the beta globin data is
shown in Fig. 12B. The quadruplicate samples of each genotype clearly group
together, most clearly seen in this case around 84-85° C. There is still some variation
within each genotype, secondary to temperature offsets between runs (note that there
is about a 0.2° C spread of quadruplicates within genotypes around 10-20%
fluorescence). This sample variation can occur between two different samples or
even between two different runs of the same sample. Different preparations,
including preparations with different salt concentrations, can also provide a
temperature offset. However, to at least a first approximation, these differences do
not affect the shape of the curve.

Temperature offsets between runs can be corrected by shifting the
temperature axis of each curve so that they are superimposed over a given
fluorescence interval. Illustratively, one sample is chosen as a standard, and the
points within the florescence interval are fit to a quadratic. For each remaining curve,
the required temperature shift for translation of each point within the fluorescence
interval onto the quadratic is calculated. Each curve is then translated by the average
shift to allow superimposition of the curves within the selected fluorescence interval.
Amplification of a heterozygote produces low-temperature melting transitions of
heteroduplexes as well as higher melting transitions of homoduplexes. If the curves
are shifted to superimpose their high temperature, homoduplex region (low percent

fluorescence), heteroduplexes may be identified by their early drop in fluorescence at lower temperatures, as seen in Fig. 12C. However, since the shape of different homoduplexes does not vary much, temperature shifting different homoduplexes may obscure any difference between them.

5   Finally, different genotypes are most easily observed by plotting the fluorescence difference between normalized (and optionally temperature shifted) melting curves. A standard genotype is first selected (illustratively, the beta-globin wild type AA is used). Then, the difference between each curve and the standard is plotted against temperature, as shown in Fig. 12D. The standard (subtracted from 10 itself) is zero across all temperatures. Other genotypes trace unique paths and can be identified by visual pattern matching. Automated methods of feature extraction may also be used to assign genotypes. Additionally, while illustrative examples use saturating dyes and heteroduplex detection, it is understood that temperature shifting and temperature difference plots can be used for genotyping when heteroduplexes are 15 not present, illustratively for use in viral genotyping wherein the genome is haploid. Examples of such high resolution genotyping include hepatitis C genotyping, human papilloma virus genotyping, HIV genotyping, and bacterial identification by ribosomal DNA amplification.

Single parameters that correlate with genotype can be devised. For 20 example, normalized curves can be used to determine the temperature at which different genotypes are, say 10% melted (90% fluorescence). This clearly distinguishes some genotypes, but not others (Fig. 12B). Alternately, the maximum slope of the curve could be used to distinguish homozygotes from heterozygotes, but different homozygotes are often similar in maximum slope. Finally, the area under 25 the difference curves (Fig. 12D) could be used to define genotype, but such curves can have similar area yet trace different paths. While a combination of parameters may prove to be effective for automated genotyping determination, this technique is well suited for visual pattern matching.

It is understood that other normalization techniques are available and 30 are within the scope of the present invention. For example, the HR-1 (Idaho Technology, Salt Lake City, UT) has a setting that will automatically adjust the fluorescence value at a predetermined temperature (illustratively a fluorescence value of 100 at 40°C), and melting curves from all samples will be aligned from the same

fluorescence value. The difference between the normalization described above and this machine-controlled normalization is that with the machine-controlled normalization, the slopes of the curve before and after the transition are not flattened.

EXAMPLE 11

Analysis of Larger Amplicons

While short amplicons often result in greater genotyping differences, LightCycler Green also may be used to genotype larger amplicons. DNA melting domains are usually about 50 to 500 bp in length, and larger amplicons, for example 500-800 bp, have multiple melting domains. A sequence alteration in one domain may not affect melting of the other domains, and the variation observed within a domain may be independent of amplicon length. Thus, while examples are provided in the 400-650 bp range, there may be no upper limit to the size of PCR product that can be scanned for the presence of sequence alterations.

Moreover, because the melting of one domain appears to be independent of the melting of other domains, an invariant domain may be used as an internal control to adjust the X-axis (temperature axis), due to instrument and/or sample run variation. Heterozygotes are distinguishable from each other and from homozygotes because the shapes of the melting curves are different. The shapes of the melting curves are defined by the stability and/or kinetic melting rates of the heteroduplexes and homoduplexes present. Because multiple melting domains are present in larger amplicons, the variation in shape may occur in any portion of the curve. By adjusting the X-axis positioning of multiple curves to overlap the invariant portion of the curve, the variable portion of the curve is much easier to discern. Alternatively, by overlapping the variable portion of the curves, if various genotypes are present, the rest of the curves will vary. X-axis adjustment alternatively could be performed by adding (1) an external control nucleic acid, or (2) a dye with a second emission wavelength that does not interact with nucleic acid but whose fluorescence is dependent on temperature (a dye with a good temperature coefficient such as Cy5) to each sample prior to PCR or to melting. Temperature-axis shifting should then be performed according to the position of the melting transition of the control nucleic acid or to the intensity profile of the control dye.

Figs. 13A and 14 illustrate two examples of analysis of larger amplicons. Fig. 13A shows amplification of a 544 bp fragment from the human 5-

Hydroxytryptamine receptor 2A (HTR2A) gene, exon 2 (accession# NM_000621.1).
The forward and reverse primers were CCAGCTCCGGGAGA (SEQ ID NO 5) and
CATACAGGATGGTTAACATGG (SEQ ID NO 6), respectively. Each 10 μl reaction
contained 50 ng of genomic DNA, 0.50 μM each primer, 1 μM LC Green, 2 mM

5   MgCl$_2$, 50 mM Tris, pH 8.3, 500 μg/ml bovine serum albumin, 0.2 mM each dNTPs,
0.4U Klentaq™ (AB Peptides, St. Louis, MO), and 88 ng TaqStart™ antibody
(CloneTech, Palo Alto, CA).

        PCR reaction conditions were as follows: 40 cycles of 92° C for 0 sec,
60° C for 2 sec, 74° C for 25 sec. After PCR amplification, the samples were cooled

10   at a programmed rate of -20° C/sec. Immediately following the rapid cooling, melting
was performed on a custom 16-bit high resolution melting instrument from 70° C to
93° C at a rate of 0.30° C/sec while continuously acquiring fluorescence.

        Duplicate samples of each genotype (CC, TC, and TT) were amplified
and analyzed, as shown in Fig. 13A. The data was normalized and temperature

15   shifted as described in Example 10, except that curves were superimposed between 10
and 20% fluorescence. Fig. 13B shows a predicted melting map of the homoduplex
and the position of the polymorphism in the lower melting domain. The experimental
data show two apparent melting domains. All genotypes are similar in the higher
melting domain. Genotypes differ in the lower melting domain, where the

20   heterozygote shows typical behavior of low melting heteroduplexes with the
heterozygote curve crossing the lower melting homozygote curve and approximation
to the higher temperature homozygote with increasing temperature.

        Fig. 14 shows difference curves for amplification of a 612 bp fragment
from the cystic fibrosis transmembrane conductance regulator (CFTR) gene, exon 10

25   (accession[#] M55115). The forward and reverse primers were
AGAATATACACTTCTGCTTAG (SEQ ID NO 7) and TATCACTATATGCATGC
(SEQ ID NO 8), respectively. Each 10 μl reaction contained 50 ng of genomic DNA,
0.50 μM each primer, 10 μM LC Green, 3 mM MgCl$_2$, 50 mM Tris, pH 8.3, 500
μg/ml bovine serum albumin, 0.2 mM each dNTPs, 0.4U Klentaq™ (AB Peptides, St.

30   Louis, MO), and 88 ng TaqStart™ antibody (CloneTech, Palo Alto, CA). PCR
reaction conditions were as follows; 35 cycles of 89° C for 0 sec, 58° C for 8 sec, 74°
C for 35 sec. Single fluorescence acquisitions were taken for each sample after the 35
sec extension. After PCR amplification, the samples were cooled at a programmed

rate of -20° C/sec. Immediately following the rapid cooling, melting was performed on a custom 16-bit high resolution melting instrument from 60° C to 87° C at a rate of 0.30° C/sec while continuously acquiring fluorescence. In this example, heterozygote differentiation was best when the middle part of the curve (30-40% fluorescence) is used for X-axis adjustment. Finally, the fluorescence of each plot was subtracted from one of the wild type plots to give the difference plots shown in Fig. 14. Each sequence alteration is clearly different from the wild type and all genotypes can be differentiated.

<div align="center">

EXAMPLE 12

Targeted detection and multiplexing

with LightCycler Green

</div>

LightCycler Green may be used as a donor to excite an acceptor dye attached to an oligonucleotide probe. Because LightCycler Green may be used at or near saturating concentrations to bind to the hybridized probe at a high density (approximately two dye molecules every three base pairs), the dye is available throughout the length of double-stranded DNA for fluorescence resonance energy transfer. A probe with an acceptor dye is added to the reaction before PCR, amplified and is detected when hybridized to the product. The binding of LightCycler Green at high density to the duplex provides favorable excitation to the acceptor dye on the probe, producing a high degree of acceptor fluorescence. Previously, dyes with a high bp/dye ratio were used and only produced low levels of acceptor fluorescence.

Multicolor experiments can be performed with multiple probes. For example, total amplicon melting can be monitored at 470 nm, the emission of a fluorescein-labeled probe could be monitored at 515, a HEX-labeled probe (that hybridizes to a different segment of DNA internal to the primers) monitored at a third wavelength, and a TET-labeled probe (that hybridizes to yet a different segment internal to the primers) monitored at a 4th wavelength. Color compensation, as is well known in the art, is used to deconvolute the overlapping four signals. The result is that the first signal can be used to scan for mutations over the whole amplicon, while the 2nd, 3rd, and 4th signals allow genotyping of smaller regions within the amplicon.

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EXAMPLE 13

Other dsDNA binding dyes

Table 1 summarizes the properties and characteristics of 37 different dyes. Twelve of the dyes tested did not produce a heteroduplex peak when a

5   heterozygous delta F508 genotype was amplified (non-operative). A heteroduplex peak was detected with 25 of 37 different dyes (operative). The strongest heteroduplex signal occurred when LightCycler Green was used, with several other dyes also showing good heteroduplex signals. Most of the dyes that revealed heteroduplexes were compatible with PCR at saturating or near saturating

10   concentrations. This correlation allows reasonable prediction of the dyes that can detect heteroduplexes by melting curve analysis. A 50% saturation provides reasonable prediction of heteroduplex detection. While missing some operative dyes, a percent saturation of about 80%, 90%, or even 99% may be used to identify dyes capable of detecting heteroduplexes.

15   Also, many of the operative asymmetrical cyanine dyes have a low bp/dye ratio at 50% saturation, particularly below 4.0 bp/dye and more particularly below 2.0 bp/dye. It was initially thought that because of this low bp/dye ratio, redistribution was prevented or minimized during the early stages of melting, and, thus, heteroduplexes were more easily detected. However, as seen in Table 1, it has

20   been found that some dyes are able to detect heteroduplexes even with a substantially higher bp/dye ratio. Furthermore, dyes with a low bp/dye ratio can detect heteroduplexes even when present in concentrations substantially below saturation levels. Thus, a low bp/dye ratio is just one factor in identifying suitable dyes for heteroduplex formation.

25   The fluorescence is preferably stronger when the molar extinction coefficient is high (>30,000). The top two dyes (in terms of heteroduplex detection), show maximal excitation at 430-455 nm, and maximal emission at 450-480 nm. These are, in general, shorter wavelengths than usually employed in the fluorescein channel of typical real-time instruments. Even so, the fluorescence signal from

30   LightCycler Green is greater than that obtained from SYBR® Green I, a surprising finding considering that SYBR® Green I has a much better wavelength match with the filters employed (Fig. 11).

TABLE 1.

	Trade name	Dye Characteristics e[1]	Ex/Em[2]	Maximum PCR Compatible dye (µM)[3]	%Sat[4]	50% saturation bp/dye ratio	dye (µM)[5]	%Het[6]
Non-operative	Ethidium Bromide	5.2K	518/605	16	12	0.15	97	None
Non-operative	SYBR® Green I	--	489/510[a]	1:5000[b]	46	--	--	None
Non-operative	SYBR® Gold	--	482/529[a]	1:40960[b]	7	--	--	None
Non-operative	Pico Green	--	472/503[a]	1:640[b]	17	--	--	None
Non-operative	SYTOX® Green	67K	484/516[a]	0.5	17	8.8	1.7	None
Non-operative	SYTOX® Orange	79K	514/539[a]	3.9	36	4.6	3.3	None
Non-operative	TOTO™-1	117K	514/533	0.4	11	17	0.9	None
Non-operative	YOYO®-1	99K	491/509	0.8	67	30	0.5	None
Non-operative	SYTO® 14	60K	517/549	3.9	76	6.8	2.2	None
Non-operative	SYTO® 21	43K	494/517	0.5	28	15	1.0	None
Non-operative	SYTO® 24	58K	490/515	1.0	68	21	0.7	None
Non-operative	SYTO® 25	57K	521/556	1.0	35	8.3	1.8	None
Operative	LC Green	--	450/469[a]	63	99	1.5	10	13.6
Operative	PO-PRO™-1	50K	435/455	50	100	2.3	6.5	11.2
Operative	JO-PRO™-1	94K	530/546	12.5	100	7.1	2.1	10.5
Operative	BO-PRO™-1	58K	462/481	3.1	75	4.8	3.1	10.2
Operative	GelStar®	--	493/527	1:1280[b]	99	--	--	8.9
Operative	SYTO® 45	43K	455/484	125	100	1.5	10.2	8.9
Operative	SYTO® 44	56K	446/471	31	86	1.4	10.5	8.3
Operative	YO-PRO®-1	52K	491/509	6.3	87	8.3	1.8	7.9
Operative	POPO™-3	146K	534/570	6.3	100	11	1.4	7.7
Operative	SYTO® 12	54K	499/522	63	100	1.7	8.7	7.5
Operative	TOTO™-3	154K	642/660	3.1	100	17	0.9	7.0
Operative	SYTO® 16	42K	488/514	12.5	99	6.0	2.5	6.6

	Dye Characteristics			Maximum PCR Compatible		50% saturation[5]		%Het[6]
	Trade name	e[1]	Ex/Em[2]	dye (µM)[3]	%Sat[4]	bp/dye ratio	dye (µM)	
Operative	Thiazole Orange	63K	510/525	16	27	0.34	44	6.0
Operative	YOYO®-3	167K	612/631	3.1	100	15	1.0	6.0
Operative	SYTO® 43	48K	436/471	125	100	0.83	18.0	5.3
Operative	SYTO® 11	75K	508/527	16	100	4.7	3.2	4.6
Operative	SYTO® 13	74K	488/509	1.0	50	8.8	1.7	4.6
Operative	SYTO® 15	55K	516/546	7.8	77	4.2	3.6	4.6
Operative	BOBO™-3	148K	570/602	3.1	85	8.3	1.8	4.6
Operative	LO-PRO™-1	102K	567/580	6.3	83	6.0	2.5	4.4
Operative	SYTO® 23	46K	499/520	3.9	70	5.4	2.8	4.3
Operative	TO-PRO®-1	63K	515/531	1.6	67	17	0.9	3.6
Operative	SYTO® 20	64K	512/530	1.5	23	4.3	3.5	2.5
Operative	BOBO™-1	114K	470/535	1.6	42	8.8	1.7	0.9
Operative	POPO™-1	93K	434/456	1.6	48	8.8	1.7	0.3

1. Molar extinction coefficient, according to manufacturer's data.
2. Fluorescence spectra either provided by the manufacturer, or (superscript a) obtained in a fluorimeter using 2.5 µM bp (100ng/60µl) of dsDNA and dye at maximum PCR compatible concentration in PCR buffer (3 mM MgCl₂, 50 mM Tris, pH 8.3, 200 µM each dNTP, 500 µg/ml BSA). Ex=excitation maxima; Em=emission maxima.
3. Maximum concentration of dye that can be present in a PCR mixture that allows amplification without significant inhibition. Some dye concentrations are expressed as dilution factors from material supplied by the manufacturer (superscript b).
4. Percent fluorescence at maximal PCR compatible dye concentration compared to fluorescence of the same dye at saturating concentration, i.e., the concentration that provides the highest fluorescence intensity possible, all in the presence of 15 µM bp DNA (100 ng dsDNA/10 µl) and PCR buffer.
5. Concentration of dye, or bp/dye ratio required to produce 50% of the maximum fluorescence intensity that is obtainable, i.e., the intensity at saturation, in the presence of 15 µM bp DNA.
6. Percentage peak area of the heteroduplex signature peak as measured with 450-490 nm excitation and 510-530 nm detection optics on the HR-1 high resolution instrument, using a melting curve of the 44 bp amplicon of the del F508 heterozygote of Example 5, obtained at a heating ramp of 0.3°C/s.

EXAMPLE 14

Preparation and use of pyrimidinium-based DNA binding dyes

Certain embodiments of dyes having the following pyrimidinium core structure have been prepared:
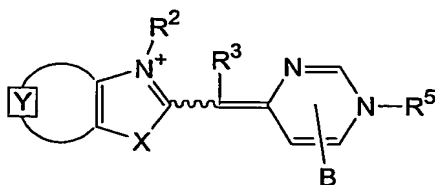
wherein Y, X, R^2, R^3, and R^5 are as defined herein for Formula I, and B is as defined in Formulae V.

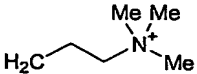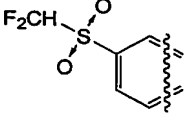While there are many ways of preparing dyes having this formula, one method is as follows:
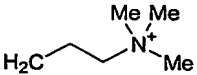

6   **7a**   **7b**

7a

or   +

7b

8   **9**
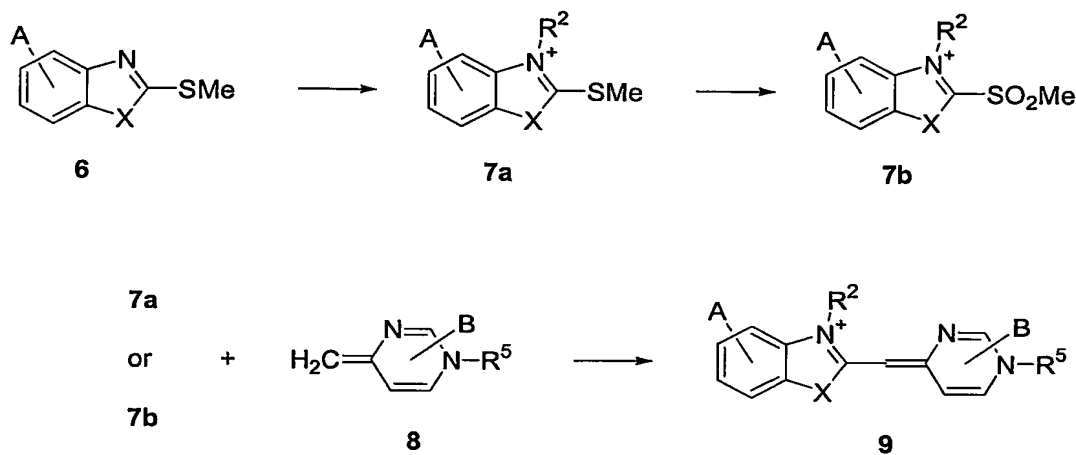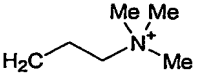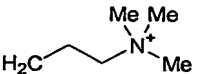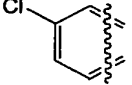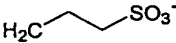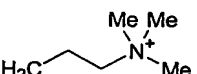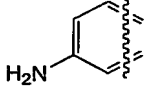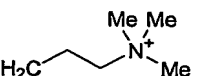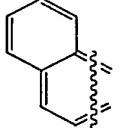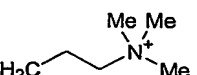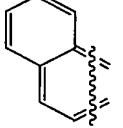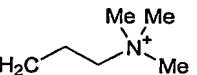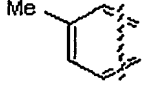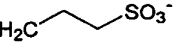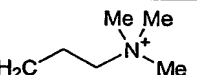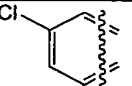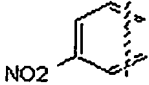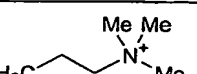
where compounds **6** are commercially available, or may be prepared by conventional methods. Compounds **7a** are prepared by alkylation of **6** at N(3) using alkylating agents such as alkyl halides, alkylsulfates, and the like, under neutral or basic conditions, including alkyllithiums, aromatic and aliphatic amines, K_2CO_3, and the like. Similarly, compounds **7a** are prepared by arylation of **6** at N(3) by aromatic coupling reactions of aromatic halides, boronates, and the like, which are catalyzed by metal compounds, such as copper, palladium, platinum, and like catalysts. Compounds **7b** are prepared from compounds **7a** by conventional oxidation, such as reactions using hydrogen peroxide, peroxy acids, including *m*-CPBA, and the like. In some cases, compounds **7a** or compounds **7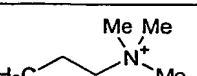b** are commercially available. Compounds **8** are commercially available or are prepared by conventional methods, such as alkylation or arylation at N(1), as described herein.

Alternatively, compounds 8 are prepared by condensation of appropriately substituted 1,3-diones and ureas or thioureas. Further, compounds 8 having leaving groups, as defined herein and including halides, alkylsulfonyls, arylsulfonyls, and the like, at C(2) may be modified by introducing nucleophiles, such as nitrogen, oxygen, and

5   sulfur-based nucleophiles, at C(2) under neutral or basic conditions. Further, compounds 8 having oxygen or sulfur nucleophilic groups at C(2) may be modified by introducing alkylating or acylating agents at the C(2) hydroxy or thiol under neutral or basic conditions. Compounds 9 are prepared by reacting compounds 7 and compounds 8 under neutral or basic conditions, as described herein.

10   Exemplary compounds having this formula were prepared as herein described, purified by HPLC using triethylamine-ammonium acetate as the mobile phase, and isolated as their corresponding acetate salts. These exemplary compounds are illustrated in Table 2.

TABLE 2.

Dye	Y	X	R²	R⁵	B
G5	(phenyl)	S	Ph	H₂C–CH₂–CH₂–N⁺(Me)(Me)(Me)	H
H5	(F₂CH–SO₂–phenyl)	S	Me	H₂C–CH₂–CH₂–N⁺(Me)(Me)(Me)	H
D6	(phenyl)	S	Me	H₂C–CH₂–CH₂–N⁺(Me)(Me)(Me)	H
E6	(phenyl)	O	Me	H₂C–CH₂–CH₂–N⁺(Me)(Me)(Me)	H
P6	(Cl–phenyl)	S	H₂C–CH₂–CH₂–SO₃⁻	H₂C–CH₂–CH₂–N⁺(Me)(Me)(Me)	H
R6	(H₂N–phenyl)	S	Me	H₂C–CH₂–CH₂–N⁺(Me)(Me)(Me)	H
Y6	(naphthyl)	O	Me	H₂C–CH₂–CH₂–N⁺(Me)(Me)(Me)	H
Z6	(naphthyl)	S	Me	H₂C–CH₂–CH₂–N⁺(Me)(Me)(Me)	H
D8	(phenyl)	S	Me	Ph	2-SH 6-Me
I5	(Me–phenyl)	S	H₂C–CH₂–CH₂–SO₃⁻	H₂C–CH₂–CH₂–N⁺(Me)(Me)(Me)	H
K5	(Cl–phenyl)	S	Me	H₂C–CH₂–CH₂–N⁺(Me)(Me)(Me)	H
L5	(NO2–phenyl)	S	Me	H₂C–CH₂–CH₂–N⁺(Me)(Me)(Me)	H
G8	(phenyl)	S	Me	Ph	2-SCH₃ 6-Me

K8		S	H_2C~~~SO$_3^-$	Ph	2-SH 6-Me
L8		S	None	Ph	2-SH 6-Me
I8		S	Me	Ph	2-SH 6-Me
M8		S	Me	Ph	2-S-pyrimidine 6-Me
N8		S	Me	Ph	2-SH 6-Ph
C8		S	Me	Ph	2-NH$_2$ 6-Me
E8		S	Me	Ph	2-OH 6-Me
F7		S	Me	Ph	2-(4,4-dimethyl piperazine) 6-Me
O8		S	Me R^3=C(O)Ph	Ph	2-SH 6-Me

Compound D6 was prepared by first reacting 4-methylpyrimidine with (3-bromopropyl)trimethylammonium bromide in acetonitrile at reflux The resulting product (compound A6) in acetonitrile was reacted with 3-methyl-2-methylsulfonylbenzothiazolium iodide (available from Aldrich) in the presence of anhydrous pyridine and triethylamine at reflux under argon.

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Compound E6 was prepared according to the general procedure used to prepare compound D6 from 3-methyl-2-methylsulfonylbenzoxazolium iodide (prepared by reacting 2-methylsulfonylbenzoxazole with dimethylsulfate) and compound A6.

5   Compound G5 was prepared according to the general procedure used to prepare compound D6 from 2-methylthio-3-phenylbenzothiazolium (Aldrich) and compound A6.

Compound H5 was prepared according to the general procedure used to prepare compound D6 from 5-difluoromethylsulfonyl-3-methyl-2-

10   methylthiobenzothiazolium methylsulfate (prepared by reacting 5-difluoromethylsulfonyl-2-methylthiobenzothiazole, available from Aldrich, with dimethylsulfate) and compound A6.

Compound P6 was prepared according to the general procedure used to prepare compound D6 from 5-chloro-2-(methylthio)-3-(3-sulfopropyl)-

15   benzothiazolium hydroxide (Aldrich) and compound A6.

Compound R6 was prepared according to the general procedure used to prepare compound D6 from 6-amino-3-methyl-2-methylthiobenzothiazolium methylsulfate (prepared by reacting 6-amino-2-methylthiobenzothiazole, available from Aldrich, with dimethylsulfate) and compound A6.

20   Compound Y6 was prepared according to the general procedure used to prepare compound D6 from 3-methyl-2-methylsulfonylnaphtho[1,2-*d*]oxazolium methylsulfate (prepared by reacting 2-methylsulfonylnaphtho[1,2-*d*]oxazole, available from Chem Bridge Product List, San Diego, CA, with dimethylsulfate) and compound A6.

25   Compound Z6 was prepared according to the general procedure used to prepare compound D6 from 3-methyl-2-methylsulfonylnaphtho[1,2-*d*]thiazolium methylsulfate (prepared by reacting 2-methylsulfonylnaphtho[1,2-*d*]thiazole, available from Specs, Rijswijk, The Netherlands, with dimethylsulfate) and compound A6.

30   Compound D8 was prepared by heating a solution of *N*-phenylthiourea and 2,4-pentanedione in HCl/EtOH at reflux. The resulting pyrimidinthione was reacted with 3-methyl-2-methylsulfonylbenzothiazolium iodide in the presence of

-52-

triethylamine in chloroform/methanol (10:1) at reflux overnight to give compound D8.

Compounds I5, K5, L5, G8, K8, L8, I8, M8, N8, C8, E8, F7, and O8 may be prepared by similar methods described above. It is expected that these dyes
5   would be useful for detection of heteroduplexes.

The pyrimidinium-based cyanine dyes described herein, illustratively G5, H5, D6, E6, P6, R6, Y6, Z6, and D8, are novel and can be used for the detection of heteroduplexes, mutation scanning and genotyping. The results of using these dyes in the detection of heteroduplexes are summarized in Table 3. It is noted that the
10   percent heteroduplex for LC Green is greater in Table 3 than in Table 1. This difference is likely due to the larger amplicon used in obtaining the data shown in Table 3.

TABLE 3.

Dye	Ex/Em[1]	Maximum PCR compatible % Sat[2]	% Het[3]
LC Green	450/469	>99 %	20.5 %
PO-PRO™-1	438/457	100 %	19.6 %
BO-PRO™-1	438/457	100 %	17.1 %
D6	457/471	92 %	23.3 %
E6	425/454	>99 %	15.0 %
P6	464/490	100 %	21.0 %
R6	453/470	>90%	15.0 %
G5	442-458/475	100 %	20.0 %
H5	444/459	100 %	22.5 %
Y6	439/477-515	100 %	21.0 %
Z6	469/494-526	100 %	13.4 %
D8	453-457/471	100 %	19.8 %

1. Excitation maxima (Ex) and emission maxima (Em) obtained in a fluorimeter using 2.5 μM bp (100ng/60μl)of dsDNA and dye at maximum PCR compatible concentration in PCR buffer (3 mM $MgCl_2$, 50 mM Tris, pH 8.3, 200 μM each dNTP, 500 μg/ml BSA). Some dyes have a range due to the broad emission or excitation peak.

2. Maximum amount of dye that can be present in a PCR mixture that allows amplification without significant inhibition, expressed as percentage of fluorescence compared to fluorescence of the same dye at saturating concentration, i.e., the concentration that provides the highest fluorescence intensity possible, all in the presence of 15 μM bp DNA (100 ng dsDNA/10 μl) and PCR buffer.

3. Percentage peak area of the heteroduplex signature peak as measured with 420-490 nm excitation and 450-530 nm detection optics, using the del F508 heterozygote melting curve obtained at a heating ramp of 0.3°C/s. The amplicon used in this set of experiments were 57bp long generated by primers GGCACCATTAAAGAAAATAT (SEQ ID NO: 23) and TCTGTATCTATATTCATCATAGG (SEQ ID NO: 24) Maximum % obtained was recorded.

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EXAMPLE 15

High Resolution Melting Curve Analysis for Genotype Comparison

Dyes of the invention can be used to determine whether any two individuals share the same alleles on a gene fragment. In the previous examples,

5   the genotype (including the exact allele, heterozygosity, and haplotype) of a reference sample was known. In some applications, the exact genotype of a reference sample need not be known, as long as high-resolution melting curve analysis makes it possible to determine whether a sample of another individual (or of unknown origin) is the same as the reference. An illustrative example is the identification of HLA

10   alleles shared among family members.

Human Leukocyte Antigens (HLA) are cell surface proteins of white blood cells and other tissues of the body which play a key role in immune recognition, and thus in transplant tolerance or rejection. Matching of HLA alleles between donor and recipient is important for organ transplant. HLA proteins form two major groups:

15   class I, and class II. Each group is encoded by multiple genes. The currently accepted techniques for determining the HLA allelotype of a tissue include serotyping with specific antibody reagents, hybridization with nucleic acid probes, and direct sequencing of the HLA genes. Because a large number of genes and loci need to be tested, the cost to determine the HLA allelotype is over $1,000 per person. Complete

20   genotyping of HLA is necessary when donor and recipient are unrelated. However there is about a 25% chance of a perfect HLA match between siblings and for this reason organ transplant between siblings is preferred when HLA matches indicate that it is possible. In this case it is only necessary to demonstrate that the donor and recipient relatives share the same HLA alleles. Determining the exact identity of the

25   shared alleles is not necessary.

Genomic DNA samples of CEPH/Pedigree Utah family 1331 were obtained from the Coriell Institute. There are 17 people across three generations in this family including four internal grandparents, two parents, and eleven children (pedigree of family 1331 is shown in Fig. 15). Two other samples with well known

30   homozygous genotypes of HLA-A BM15(0101) and BM16(0202) were also obtained from Coriell.

Amplification of two exons of the HLA-A gene were performed as follows: HLA class I genes are so similar over of the length of their coding exons that

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it is difficult to design PCR primers that amplify only the HLA-A gene and not the related class I genes. A nested PCR strategy was adopted in which an initial round of PCR specifically amplified a large (948bp) fragment of the HLA-A gene followed by secondary amplification of that product using internal primers. The primers used in

5 the first PCR hybridized to HLA-A intron 1 (forward primer 5'-GAAAC(C/G)GCCTCTG(C/T)GGGGAGAAGCAA (SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 12)) and intron 4 (reverse primer 5'-TGTTGGTCCCAATTGTCTCCCTC (SEQ ID NO 13)). In the secondary PCRs the forward primers 5'AGCCGCGCC(G/T)GGAAGAGGGTCG (SEQ ID NO 14, SEQ

10 ID NO 15) and reverse primer 5'GGCCGGGGTCACTCACCG (SEQ ID NO 16) were used to amplify a 335bp segment of HLA-A exon 2. The forward 5'CCC(G/A)GGTTGGTCGGGGC (SEQ ID NO 17, SEQ ID NO 18) and reverse primer 5'ATCAG(G/T)GAGGCGCCCCGTG (SEQ ID NO 19, SEQ ID NO 20) were used to amplify a 366bp fragment of HLA-A exon 3. In the primer sequences of this

15 example, (N/N') represents that the primer is a mixture of nucleotide sequences having equal percentages of N and N' at that position. For example, the forward primer for the 335bp segment of HLA-A exon 2 contains an equal mixture of two nucleotides, with either a G or an A at the fourth position, as represented by SEQ ID NO 17 and SEQ ID NO 18. The forward primer for the HLA-A intron 1 has two such

20 sites, and thus is an equal mixture of four nucleotides, as represented by SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11 and SEQ ID NO 12.

All PCRs were performed in glass capillaries using the Roche LightCycler®. The initial PCR contained 0.5 μM forward and reverse primers, 50ng genomic DNA in a buffer of 3mM Mg^{++}, 50mM Tris-HCl pH 8.3, 500μg/ml BSA and

25 20 μM of dye D6 in 10μl. Cycling conditions were 94°C for 20 s followed by 40 cycles of 94°C 1 s, 62°C for 0 s, 72°C for 1 min. The secondary, nested PCRs contained 0.25 μM forward and reverse primer, 1/10000 of first PCR product in the same buffer containing 2mM Mg^{++}. Cycling conditions were 94°C for 5 s followed by 25 cycles with 94°C 1 s, 65°C for 0 s, 72°C for 8 s.

30 After the secondary amplification the glass capillaries were transferred to the high resolution melting instrument HR-1, and a melt was performed. The sample was heated from 60°C to 95°C at a rate of 0.3°C/s and fluorescence (450 excitation/470 emission) and temperature measurements were acquired every 40 s

(Figs. 16A-B). The nested sequencing products were sequenced by the ABI 3700. Sequencher version 4.0 was used for the sequence analysis.

Concordance of melting curve analysis and sequencing results were determined as follows: Melting curve analysis of the exon 2 and exon 3 PCR
5   products amplified from the 17 members of the CEPH/Pedigree Utah family 1331 clustered in six different groups (Figs. 16A-B). This suggested that there are six different HLA-A genotypes in this family. The exon 2 and exon 3 PCR products were sequenced, and the results confirmed the melting curve analysis, identifying the six genotypes as: HLA-A 02011/3101 (herein referred to as genotype AB) for family
10   members 1, 4, 7,12; HLA-A 3101/2402101 (genotype BC) for family members 3,5,6,11,17; HLA-A 02011/2402101 (genotype AC) for family members 2,9,10,16, HLA-A 02011/03011 (genotype AD) for family members 13, 14; HLA-A 02011/02011 (genotype AA) for family member 8 and HLA-A 2402101/01011 (genotype CE) for family member 15 (Results for exon 2 is shown in Figs. 16A-B).
15   In some cases, the amplification products from siblings may show identical or nearly identical melting curves despite having different genotypes. In such cases mixing the genomic DNA from the two siblings before the initial PCR followed by the two amplification steps and melting curve analysis can differentiate identical from non-identical genotypes. In particular if the siblings have identical
20   genotypes, the mixed melting curve will be identical to those performed separately. If siblings have different genotypes then the mixed melting curve will be different from that of the individual melting curves. Mixing experiments within each group confirmed that the members of each group shared identical genotypes.

Another example of the mixing analysis technique was demonstrated
25   by two homozygous samples BM15 (0101) and BM16 (0201). In this case, the two alleles have a total of 15 nucleotide differences spread over the length HLA-A exon 2, but they show similar melting curves. The melting curve of the mixed samples was significantly shifted to the left (lower melting temperature) due to the 15 mismatches present in the heterohybrids generated in the mixed sample PCR from HLA-A exon 2
30   (see Fig. 17).

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EXAMPLE 16

Monitoring amplification in real-time with saturating dyes

A 60 bp fragment of the HTR2A gene was amplified with forward and reverse primers ACCAGGCTCTACAGTAA (SEQ ID NO 21) and

5   GTTAAATGCATCAGAAG (SEQ ID NO 22), respectively. Amplification was performed using the reagents described in Example 11 but with modifications to the cycling parameters, which were 95°C, 0 s; 62°C, 2 s; 74°C, 20 s using the LightCycler®. Various concentrations of SYBR® Green I, GelStar®, and SYTO® 16 were independently present in the reaction mixture. Fluorescence data were acquired

10   once each amplification cycle, up to 40 cycles. Fluorescence crossing points (Cp), calculated as the second derivative maximum of the amplification plot (cycle number plotted on the x-axis against fluorescence intensity on the y-axis), were obtained as follows:

TABLE 4.

Dye present in reaction	Dilution/Concentration	Cp
SYBR® Green I	1:2,500	No amplification
	1:5,000	26
	1:10,000	26
	1:20,000	Signal too weak
GelStar®	1:640	No amplification
	1:1,280	29
	1:2,560	28
	1:5,120	31
	1:10,240	31
	1:20,480	Signal too weak
SYTO® 16	25 μM	No amplification
	12.5 μM	30
	6.25 μM	29
	3.1 μM	31
	1.5 μM	33
	0.8 μM	Signal too weak

15

The Cp value, which represents the cycle number at which signal rises above background, is expected to increase when inhibitors present in the reaction affect the efficiency of amplification. Under the conditions of these experiments, however, inhibition by increasing amounts of dye resulted not as a gradual increase in Cp, but as a sudden and complete elimination of amplification. Due to the small size of the amplicon (which results in a lower signal compared to larger amplicons), SYBR®

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Green I dye could only be used in the range of two-fold concentrations for real-time monitoring. In contrast, GelStar® and SYTO® 16 could be used within a range of eight-fold concentration. It is contemplated that many saturating dyes have a wide range of concentration that can be used in real-time monitoring of amplification.

5   The foregoing discussion discloses and describes merely exemplary embodiments of the present invention. One skilled in the art will readily recognize from such discussion, and from the accompanying drawings and claims, that various changes, modifications and variations can be made therein without departing from the spirit and scope of the invention as defined in the following claims.

10   All references cited herein are incorporated by reference as if fully set forth.